FURTHER CHARACTERIZATION AND FUNCTION OF UTEROFERRIN FROM UTERINE FLUSHINGS AND ALLANTOIC FLUID OF PIGS

By

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KEY TO ABBREVIATIONS

A.F.	allantoic fluid
ATP	adenosine triphosphate
BSA	bovine serum albumin
CL	corpus luteum
CMC	carboxymethyl cellulose
Con A	Concanavalin A
DEAE-C	Diethylaminoethyl cellulose
DTT	Dithiothreitol
HSA	human serum albumin
LAP	luceine aminopeptidase
Lf	lactoferrin
LH	lutenizing hormone
MES	Morpholinoethane Sulfonic Acid
MOPS	Morpholinopropane Sulfonic Acid
NEPHGE	Non-equilibrium pH gradient electrophoresis
p.c.	post-coital
PEG	polyethylene glycol
pNPP	p-nitrophenyl phosphate
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
TCA	Trichloroacetic acid
TEMED	N, N, N', N' - Tetramethyl-ethylene diamine

Tf	transferrin
TPCK	L-1-Tosylamide-2-phenylethyl chloro-methyl ketone
Tris-HCl	Tris-(hydroxymethyl)-aminomethane, hydrochloride
2D-PAGE	Two dimensional polyacrylamide gel electrophoresis
Uf	uteroferrin

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By

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Uteroferrin, a purple progesterone-induced glycoprotein with acid phosphatase activity, is secreted by the surface and glandular epithelium during pregnancy. It is taken up by the placenta and accumulates in allantoic fluid. In this study the molecular weight of uteroferrin using SDS-gel electrophoresis has been confirmed to be about 35,000 and the iron content, determined colorimetrically to be about 0.165% on a dry weight basis, corresponding to 1 iron atom per polypeptide chain. An amended extinction coefficient for uteroferrin of $3.1 \times 10^3\ M^{-1}$ at 545 nm is presented.

Sterile allantoic fluid incubated at 37° shows a time-dependent loss of iron and acid phosphatase activity from uteroferrin. The protein is degraded proteolytically and becomes less immunoprecipitable, although this appears to follow rather than precede the loss of iron. Apo-uteroferrin is rapidly destroyed in allantoic fluid indicating that removal of iron from the protein renders it more prone to proteolytic attack. The majority of iron from ^{59}Fe-uteroferrin was transferred to

a protein with some properties of transferrin during the decline of acid phosphatase activity. The presence of transferrin, other fetal serum proteins, and maternally derived proteins in allantoic fluid was demonstrated by two-dimensional electrophoresis.

A radioimmunoassay for uteroferrin was developed, its validity established and the uteroferrin content of allantoic fluid throughout pregnancy was measured. Amounts were maximal at Days 60 and 75, with a content of 830-1400 mg per pregnant gilt.

In an in vivo series of experiments, ^{59}Fe-labelled uteroferrin and transferrin were introduced into the allantoic sacs of pregnant gilts and the disposition of the isotope followed. There was an approximate first order loss of iron from uteroferrin with Fe recovered in a protein identified as transferrin. However, Fe was also found to be concentrated in fetal hemoglobin and in fetal liver and spleen.

The half-life of iron loss from uteroferrin was about 15 hours, implying that turnover of uteroferrin in allantoic fluid can account for the transfer of about 1.1 to 1.9 mg of iron per day from the mother to the fetuses at Day 60. When ^{59}Fe-transferrin was introduced into allantoic fluid, the distribution of iron in the fetus was similar to that observed with ^{59}Fe-uteroferrin. Thus, it is likely that the tissue distribution from Fe-uteroferrin is a consequence of transfer of the iron to transferrin.

Iodination of uteroferrin and transferrin allowed the fate of the polypeptide to be followed in vivo. A portion of the ^{125}I-uteroferrin injected into the allantoic sac was recovered in fetal tissues but was largely non-precipitable with trichloracetic acid. The ratio of ^{125}I in fetal serum to that in allantoic fluid was very low. With

^{125}I-transferrin, there was evidence for equilibration with the fetal blood from which most was recovered intact. By contrast, transferrin introduced into maternal blood did not cross the placenta in an intact form.

When ^{125}I-uteroferrin was incubated in allantoic fluid, the protein was cleavaged to yield two fragments of approximate molecular weight 22,000 and 14,500. Using trypsin and chymotrypsin as model proteases, similar internal cleavage occurred. Thus, it is likely that the uteroferrin structure contains a protease sensitive region or loop. Apouteroferrin, however, is rapidly degraded by trypsin.

Studies in vitro have demonstrated that ^{59}Fe can be transferred from uteroferrin to transferrin provided that certain low molecular chelators (citrate, pyrophosphate, ATP and ascorbate) are present in the reaction mixture. Of these, ascorbic acid was most efficient and, because of its presence in allantoic fluid, the likely intermediary in iron transfer in vivo. The iron did not appear to be reduced to Fe II during this transfer.

CHAPTER 1
INTRODUCTION

General Aspects of the Pig Reproductive Tract

The reproductive organs of the female pig, Sus scrofa, are composed of the following: the ovary, the oviducts, the cervix, the uterus, the vagina, and external genitalia. Each system has specific functions in reproductive events which result in delivery of viable neonates (Figure 1-1). The ovary performs both an exocrine function (egg development and release) and an endocrine function (production of estrogen and progesterone). At any time, the pig has 10-25 follicles in different stages of maturation or atresia on its surface giving the appearance of "a cluster of grapes." As estrus begins, the ovary releases 10-20 eggs and the ruptured follicles become filled with blood and are called corpora hemorrhagica. By Day 4, luteal cells begin to secrete progesterone and undergo hypertrophy primarily and hyperplasia, to a lesser extent, so that the antrum of each former follicle becomes known as a corpus luteum (CL). In the nonpregnant animal, progesterone synthesis decreases and CL regresses after Day 15 to become hormonally inactive corpora albicantia.

The oviduct functions uniquely to transport the released ova down from the ovarian end and spermatozoa up from the tubo-uterine junction. It is within the oviduct that fertilization and early cleavage of fertilized ova takes place.

The porcine uterus consists of two uterine horns (cornua), each about 1.5 meters long, which are connected at their distal ends by a

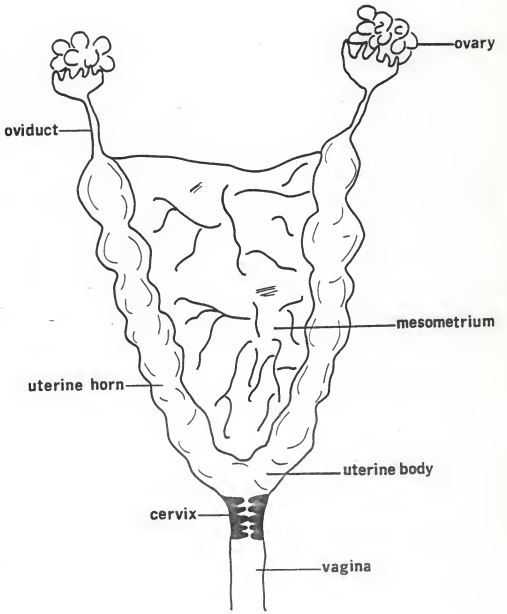


Figure 1-1. Diagram of the Porcine Female Reproductive Tract

small uterine body, and the cervix and is of the type of uterus referred to as bicornuate. The two uterine horns are connected and supported by a highly vascularized mesometrium which holds the horns in a convoluted and folded position. Histologically, the uterus consists of an inner layer, the endometrium, a highly glandular and vascularized structure having an epithelial lining of the lumen over a bed of connective tissue. The myometrium is the muscular portion of the uterus, having two layers of smooth muscle, one longitudinal and the other circular. The uterus serves a number of functions, e.g., transport of spermatozoa to the oviduct, sperm capacitation in endometrial secretions, production of a luteolytic agent in the event of an infertile mating, nutrient supply to the developing blastocyst in pregnancy and after placentation, direct transfer of nutrients (histotroph) from the endometrial glandular and surface epithelium to the conceptus and finally expulsion of the fetus at term.

The functions of the cervix are to present a barrier to bacteria, aid in transport of sperm, and permit expulsion of the fetus.

The vagina serves to aid in copulation and as a passageway for fetal delivery.

Female pigs are polyestrous, that is, they have estrous cycles continuously throughout the year. The average length of each estrous cycle is 21 $\pm$ 2 days. The onset of estrus (Day 0) starts with the LH surge. This is preceded 36-48 hours by a rapid increase in serum estrogen levels (proestrous). Ovulation occurs 40 to 44 hours after the LH surge (Day 0). The endometrium, which is under control of follicle-secreted estrogen at proestrus, becomes more vascularized and the surface and glandular epithelium undergo proliferation with some

growth, but remain nonsecretory. As a result of ovarian changes at estrus, ovulation and corpus luteum development, the endometrium comes under the influence of CL-produced progesterone starting at Day 4 of the cycle. Progesterone levels increase from less than 5 ng/ml to 20-40 ng/ml by Day 12-14 according to Anderson (1978), Hansel et al. (1973) and Zavy (1979). The endometrium increases in thickness and surface and glandular epithelium becomes highly columnar and the uterine glands reach maximum development. The uterine glands are larger, more coiled and branched and the epithelium is actively secretory during the luteal phase. These cyclic changes occur whether or not fertilization is achieved. By Day 16, progesterone levels fall to basal levels if pregnancy is not achieved and a new proestrus period begins on about Day 18 and leads to initiation of a new estrous cycle.

Genital Tract Secretions

Bonnet (1882) stated that Aristotle (384-322 B.C.) and William Harvey (1578-1657) believed that uterine milk or histotroph was involved in nurturing the conceptus. Interest in the composition and secretory pattern of genital tract secretions was reported by Amoroso (1958) to have dated from as early as 1842 by Prevast and Moriss, Schlossberger (1855), Gamgee (1864), and Bonnet (1882). These classical investigations revealed that uterine milk (histotroph) consisted of proteins, fat globules, trace amounts of minerals, and cellular debris.

That uterine secretions play a role in intrauterine embryonic/fetal development has been studied in many species (including the mouse, rat, cow, sheep, pig, and others). Consideration has been given to sperm motility factors, sperm capacitation factors and fertilization, shedding

the zona pellucida, expansion and elongation of the blastocyst, blastocyst development, production of a luteostatic signal for the so-called "maternal recognition of pregnancy" and luteolytic agents, and factors required for pregnancy maintenance. Evidence that uterine secretions are essential for embryonic development beyond the early blastocyst stage has been examined by restricting embryos to their oviductal environments by ligation at the tubo-uterine junction. Development is arrested at the early blastocyst stage in mice (Kirby, 1962; Orsini and McLaren, 1967), rats (Alden, 1942), rabbits (Westman et al., 1931; Pincus and Kirsch, 1936; Adams, 1958), and sheep (Wintenberger-Torres, 1956). In addition, many of these restricted embryos underwent degeneration within the oviduct. Murray et al. (1971) reported that pig embryos so restricted developed only to the 4- to 32-cell stage, but no further. Pope and Day (1972), however, reported that pig embryos in the ampulla of the oviduct were capable of development to the blastocyst stage, but no further.

The morphological transformation of the endometrium is characterized by preparation of endometrial cells for synthesis and secretion of products during the estrous cycle and early pregnancy. These transformations are clearly under the control of estrogen and progesterone and can be imitated by classical endocrine substitution of ovarian hormones in the castrated animal. However, when a hormonal imbalance results, transformation of epithelial and stromal areas is altered and the sequence of secretion patterns are disturbed as indicated by failure of implantation. When endometrial transformation results in normal secretion, typical protein patterns, that is, the characteristic proteins found at each day of the preimplantation

period, are obtained. Beier et al. (1971), Beier et al. (1972), and Beier (1974) reported that rabbits injected post-coitally (p.c.) at 6 h and 24 h with estrogen exhibit a "delayed" uterine secretory pattern of 4 to 5 days. Blastocysts in the estrogen-treated females failed to develop beyond the blastocyst stage. However, when 4-day old blastocysts were transferred to the estrogen-treated females on day 8 p.c., approximately 40% of these embryos survived, suggesting a return to synchrony (and normal protein secretion) is necessary for survival. Further, Courrier and Kehl (1932) reported that p.c. administered estrogens lead to a delay in normal endometrial histological transformation. Modification of the time of injection and the dosage regimen of estrogen by Adams (1973; 1976) has confirmed Beier's work.

Another treatment described by de Visser (1979) that leads to asynchronous uterine and embryonic development in the rabbit is precoital injection of progesterone which allows prefertilization stimulation of uterine secretions to be advanced. This treatment results in normal egg development during oviductal passage, but a remarkable degeneration of blastocysts was seen in the uterine lumen. McCarthy (1977) also performed this type of experiment and found that rabbit intrauterine protein patterns were advanced and did not match expected reproductive stages relative to that of the blastocysts.

Early work with embryo transfer in sheep by Rowson and Moor (1966) and in cows by Rowson et al. (1972) showed a rather stringent requirement for synchrony between donor and recipient animals. The implication was that the uterine environment changes with time and compositional changes are related to embryonic survival.

In prepubertal gilts induced to ovulate, maintenance of pregnancy is only about 50% successful with or without supplementary progestins

and/or estrogens as described by Ellicott et al. (1973), Neville et al. (1971), and Rampacek (1975). Murray and Griffo (1976) suggest that the inability of prepubertal gilts to secrete all of the uterine specific proteins may be the cause of pregnancy failure.

Uterine secretions, therefore, constitute the environment that the early embryo encounters and, as such, must be of importance in mammalian reproduction, particularly before implantation.

Available data concerning composition of mammalian uterine secretions are extensive. Some examples of the many studies and species examined are: man (Beier et al., 1970; Wolf and Mastroianni, 1970; Roberts et al., 1976a; Maathius and Aitken, 1978), rat (Dupont-Mairesse and Galand, 1975; Umpathysivam and Breed, 1976; Surani, 1976), baboon (Peplow et al., 1973), mink, dog, armadillo, and black bear (Daniel and Kirshnan, 1969), seal (Daniel, 1972), golden hamster (Noske and Daniel, 1974), sheep (Roberts et al., 1976; Cerini et al., 1976), cow (Roberts and Parker, 1974a ; Roberts et al., 1976a; Laster, 1977), mare (Zavy et al., 1976; Zavy et al., 1979), and various marsupials (Renfree, 1973; 1975). Two other species, the rabbit and the pig, have been studied more thoroughly and from each a major uterine protein has been isolated which is apparently under ovarian steroid control.

In these many systems, attempts have been made to analyze the composition of the uterine secretory fluids. In general, they have been analyzed for their physical and chemical properties, electrolyte composition, carbohydrate, lipid, and protein concentrations, similarity to serum components, hormone binding properties and enzyme activities. The major constituents of many of the genital tract secretions have been shown by numerous investigators, using immunological and

electrophoretic techniques, to be identical to serum proteins. Some that have been identified include albumin and transferrin and many others remain to be characterized positively.

Uterine secretions have been shown to have a wide range of enzyme activities and their appearance in uterine secretions is generally related to the secretory patterns induced by ovarian steroid hormones.

Phosphatases (acid and alkaline) have been reported in the rat by Ringler (1961), the sow (Chen et al., 1975), the mare (Zavy et al., 1979), and the cow (Schultz et al., 1971; Dixon and Gibbons, 1979).

Schwick (1965) identified neuraminidase activity and Kirton and Hafs (1965) reported the presence of β-amylase in rabbit uterine fluid. Ringler (1965) also identified β-glucuronidase activity in the rat. Roberts and Parker (1974a)demonstrated the very active glycosidases, β-N-acetyl-glucosaminidase, β-N-acetylgalactosaminidase and α-fucosidase, in uterine flushings from the cow during early pregnancy. Other enzymes found in serum were also shown to be present, but not significantly elevated above serum activities. Roberts and Parker (1974a)further demonstrated increased activities (above basal serum activities) of certain glycosidases in rabbit luminal fluid, especially in pregnant, but also in nonpregnant animals. Roberts et al. (1976a) identified glycosidase activity in genital tract secretions in the ewe that parallel those of the cow. Additionally, Roberts et al. (1976a) reported glycosidases in the human genital tract.

Protease activity has been demonstrated in uterine secretions of rabbits (Kirchner et al., 1971) and mice (Pinsker et al., 1974). Leucine amino peptidase (LAP) has been purified from porcine uterine flushings and its properties studied (Roberts et al., 1976b;Basha et al., 1978)

and it is apparently under the control of progesterone. Denker (1980) has also described LAP in rabbit uterine lumen. Further, Roberts et al. (1976b) demonstrated cathepsin (B_1, D_1, and E) in flushings from progesterone-treated animals.

Lysozyme is secreted into the uterine lumen of pigs in response to progesterone according to Roberts et al. (1976b). Its presence has been reported in human cervical secretions (Shumacher, 1974) and in placenta and fetal fluids of humans during pregnancy (Sutcliffe, 1975; Galask and Snyder, 1970). Presumably,it functions as a bacteriostatic agent. Phosphohexose isomerase activity was found in uterine flushings by Zavy, Roberts, and Bazer (unpublished).

Other proteins which do not have enzymatic activity have been identified in uterine flushings. Adams et al. (1981) have shown the presence of a retinol binding protein and have evidence for a retinoic acid binding protein in pig uterine secretions. Additionally, lactoferrin has been observed in uterine secretions of the cow (Dixon and Gibbons, 1979) and human (Masson et al., 1968) and it is most likely under control of progesterone.

a. Rabbit

Schwick (1965) demonstrated a changing protein pattern in uterine washings from preimplantation stages of the rabbit using acrylamide gel electrophoresis and agar gel electrophoresis. Subsequently, Beier (1966; 1967) described a uterine protein, uteroglobin, which was independently called blastokinin by Kirshnan and Daniel (1967). This was shown to be the predominant protein in rabbit uterine secretions during the preimplantation period in normal pregnancy and at equivalent stages in pseudopregnancy by Beier (1966; 1967; 1968a), Kirshnan and

Daniel (1967), Kirshner (1969), Urzua et al. (1970), Beier et al. (1970), Beier et al. (1971), and Daniel (1971). Uteroglobin has a characteristic pattern of secretion in early pregnancy, being detectable by Day 3, reaching a peak on Days 5-6 and disappearing on Day 10 according to Urzua et al. (1970), Arthur et al. (1972), Krishnan et al. (1967), and Bullock and Connel (1973). In addition to uterine secretions, uteroglobin is found in blastocyst fluid (Beier, 1966; 1967; Hamana and Hafez, 1970; Petzoldt, 1974). However, it is not detected in the blastocyst when grown in culture from the 2-cell stage as determined by Beier and Maurer (1975).

It is apparent that progesterone of luteal origin or exogenously administered (Beier, 1968b; Beier et al., 1970; Urzua et al, 1970; Arthur and Daniel, 1972; Bullock and Willen, 1974) is responsible for the endometrial epithelial cells to synthesize uteroglobin and it can be found in epithelial cells as well as in their secretory products. In addition, Beier and Beier-Hellwig (1973) have shown synthesis of uteroglobin to be induced experimentally in ovariectomized rabbits by progesterone alone or synthetic progestins, including chlormadinone acetate, D, L-Norgestral, and 17 α-hydroxyprogesterone caproate. Uterine flushings of these treated animals revealed copious amounts of uteroglobin. In estrogen-treated castrated does, uteroglobin is present in detectable amounts by immunohistochemical means in the epithelial cells as Kirshner (1976) has shown, but it is not released into the uterine lumen. No release was obtained by Beier (1974) using very high dosages of estrogen. The release by progesterone is to a certain extent dose-dependent, the most effective dose being 0.6 mg - 3.0 mg/day/2.5-3.0 kg. animal. Higher doses have little additional effect according to Arthur and Daniel (1972) and Beier (1976).

When estrogen is administered during the first three days post coitum, as mentioned previously, secretion of uteroglobin is delayed. This provided a clue to possible control of uteroglobin synthesis and secretion. Arthur and Daniel (1973) have shown that daily administration of progesterone to intact or ovariectomized rabbits resulted in persistence of uteroglobin synthesis beyond Day 10, whereas in normal pregnancy this protein had virtually disappeared from pregnant animals. Further, Challis et al. (1973) showed that in pregnant rabbit plasma progesterone is still rising at Day 10 so that complete and total regulation by progesterone alone cannot account for the normal pattern of uteroglobin secretion. Experiments by Beier (1968b), Beier et al. (1971), and Bullock and Willen (1974) further suggest that termination of uteroglobin synthesis may be under estrogen control. Whether it is the actual levels of those steroids or whether it is the progesterone/estrogen ratio that is more important is unknown at this time.

The abundance of uteroglobin, approximately 50% of the secreted uterine proteins, has allowed its messenger RNA to be isolated and translated in vitro by Beato and Nieto (1976), Levey and Daniel (1976), Bullock et al. (1976), and Atger and Milgrom (1977). Their results indicate that uteroglobin is synthesized de novo in the uterus and that progesterone stimulates its synthesis. In addition, uteroglobin m-RNA from the endometrium reached a maximum on about Day 4 of pregnancy and declined to nonpregnant levels by Day 8, a pattern similar to that of uteroglobin secretion. Further, data from the cell-free synthesizing systems indicated that uteroglobin is synthesized as a precursor protein (preuteroglobin) containing a 10-20 amino acid sequence not normally found on the "mature" molecule.

Recently, Beier et al. (1975), Beier (1977), Beier et al. (1978), Nöske and Feigelson (1976), and Feigelson et al. (1977) have shown a protein present in the male genital tract, the respiratory tract (bronchial secretions), and the digestive tract that is identical to uteroglobin by immunological analysis. The concentration in the lung and other non-uterine tissues is lower than that in the uterus and cannot be increased by estrogen or progesterone administration (Feigelson et al., 1977; Torkkeli et al., 1978), as can the uterine protein or by pregnancy (Daniel and Crowder, 1977). Torkkeli et al. (1978) isolated uteroglobin from uterine secretions and uteroglobin-like protein from lung and compared their biochemical and immunological properties. The two proteins had: (1) the same molecular weight (approximately 13,000) and the presence of two subunits of approximately 7,000 each; (2) identical behavior in polyacrylamide gel electrophoresis under non-denaturing and denaturing conditions; (3) the same isoelectric point; (4) lack of carbohydrate in the molecule; (5) similar amino acid composition; (6) lack of tryptophan; (7) the same N-terminal amino acid (glycine); and (9) immunological identity. In contrast to ovarian steroid regulation of uterine uteroglobin, the uteroglobin-like protein in lung has been shown by Feigelson et al. (1977), Torkkeli et al. (1978), and by Savouret et al. (1980) to be responsive to cortisol and dexamethasone, which have no effect on uterine uteroglobin secretion. Testosterone and its derivatives, however, significantly increased both lung and uterine proteins.

Savouret et al. (1980) examined the properties of uteroglobin messenger RNA from both lung and endometrium and judged them to be identical in terms of synthesis of a peptide of similar molecular weight, N-terminal 21 amino acid extensions, and immunological properties.

The role of uteroglobin in the rabbit reproductive tract is not known nor has a function been given to the uteroglobin-like protein in other tissues. While its synthesis and secretion in the uterus is apparently controlled by progesterone, it binds progesterone weakly as does the similar protein from lung and other tissues, but the latter synthesis is not under progestational control. The role of uteroglobin in promoting blastocyst development, as proposed by Krishnan and Daniel (1967), could not be confirmed by Maurer and Beier (1976). In addition, neither of these actions would seem to be its primary or sole biological function if its distribution in the male genital tract and other nonreproductive tissues is considered.

There is evidence that uterine secreted proteins, some of which are serum derived, permeate into blastocyst fluid, cells and coverings. Uteroglobin has been found within tissues and fluids of expanded blastocysts, further implying some role of the protein in the reproductive process. Beier and Mootz (1978) have suggested a protease-inhibitor function to regulate either uterine or embryonic proteases.

b. Pig

Proteins in pig uterine secretions change both quantitatively and qualitatively during the estrous cycle. Murray et al. (1972) have shown that recoverable protein from uterine flushings increase from a constant of about 10 mg at Days 2-9 to a maximum of approximately 50 mg on Day 15. After this time, protein decreases to levels observed before Day 10 of the estrous cycle in nonpregnant animals. Sephadex G-200 gel filtration chromatography revealed three protein fractions which were present throughout the estrous cycle. Two additional protein fractions (IV and V) were found not to be present prior to Day 9 or after Day 16. Fraction IV, containing a purple-colored protein, first appears on about

Day 12 and has an estimated molecular weight of 45,000. Fraction V appeared as early as Day 9 and had an estimated molecular weight of 20,000. Squire et al. (1972), using polyacrylamide disc gel electrophoresis to analyze swine uterine protein secretions, revealed that the protein components found between Days 2 and 12 exhibited similar patterns, some corresponding to serum proteins. Fraction IV consisted primarily of a single strongly basic glycoprotein (PAS positive), later named uteroferrin (Buhi et al., 1979). Fraction V consisted of at least six acidic proteins which were not found between Days 2 and 11 of the estrous cycle.

The increase in total protein, the appearance of uteroferrin (Fraction IV) and Fraction V, and the rapid decline of both total protein and specific fractions coincides with the pattern Hansel et al. (1973) described for development and subsequent regression of corpora lutea in nonpregnant pigs. This suggested a progesterone influence on both protein quality and quantity in uterine secretions. Knight et al. (1973) reported that the quantity of total recoverable uterine protein increased and the appearance of uteroferrin and Fraction V required exogenous administration of progesterone to Day 4 ovariectomized gilts. Estrogen had no effect on secretory activity. Further studies by Knight et al. (1974) established a positive dose-response relationship between progesterone dosage and quantity of uterine protein recovered and that estrogen in low doses (while ineffective alone) acts synergistically with progesterone to increase total recoverable protein, but higher doses of estrogen were inhibitory as confirmed by Frank et al. (1978). Thus, uteroferrin appears to be induced by progesterone.

Uteroferrin was purified by Chen et al. (1973) from uterine flushings by ion-exchange chromatography on CM-cellulose and Sephadex G-100 gel filtration. A further step involving linear salt gradient elution (0-0.5 M) from CM-cellulose at pH 8 produces a protein free from impurities. Chen et al. (1973) further confirmed that uteroferrin is strongly basic (pI - 9.7) and provided the molecular weight estimate of 32,000. It contains large amounts of basic amino acids and is 12.5% carbohydrate by weight. An apparently identical protein (by physical, chemical, and immunological properties) was isolated by Bazer et al. (1975) from allantoic fluid of pregnant pigs between Days 30 and 100 of gestation. Uteroferrin begins to accumulate in allantoic fluid after Day 30, reaching a maximum around Day 60-70 and then declining. At midpregnancy (Day 50-60) over 0.5 gm uteroferrin can be isolated from a single animal while ovariectomized animals and pseudopregnant animals receiving progesterone will yield about 0.25 gm of uteroferrin as determined by Schlosnagle et al. (1974).

Chen et al. (1973) used an antibody to uteroferrin to demonstrate that the protein is uterine specific and not found in maternal serum or other tissues. Subsequently, Chen et al. (1975), using fluorescent-labelled affinity purified specific antibody, studied its synthesis and distribution. They showed that uteroferrin is synthesized and secreted by endometrial surface and glandular epithelium of the uterus and during pregnancy is taken up at special structures called areolae (Brambel, 1933). Areolae are involved in absorption of nutrients and only form where the chorion (placenta) lies in direct apposition to the opening of a uterine gland. Chen et al. (1975) also showed that uteroferrin synthesis is dependent on the progesterone status of animals. Fluorescent antibody studies demonstrated that

animals receiving no progesterone or estrogen alone have little or no detectable fluorescence in epithelial cells of uterine glands. This is in contrast to the estrogen effect on rabbit uterine epithelial cells where Kirshner (1976) has shown some uteroglobin synthesis by immunohistochemical techniques. Further, Basha et al. (1979; 1980), using organ explant culture techniques with labelled amino acids, demonstrated that *in vitro* explants of pregnant and pseudopregnant endometrium secrete labelled uteroferrin into the medium and that the secretions *in vitro* are dependent upon the hormonal status of the animal supplying the tissue. Rates of secretion by endometrial explants were greatest at Days 60 and 75 of pregnancy.

Thus, while it has been established that progesterone is required for uteroferrin production, it may be that estrogen is important in modulating progesterone's role, i.e., either synergistically controlling synthesis and secretion or, at high levels, inhibiting uteroferrin synthesis and/or secretion. Evidence for this is provided from the work of Anderson (1974), Guthrie et al. (1972), and Perry (1976), who show increasing estrogen levels in early pregnancy from Day 12 to Day 30 followed by a rapid decrease to about Day 35 according to Knight et al. (1977). At this same time progesterone decreases between Days 15 and 30 and Frank et al. (1978) indicated that uteroferrin decreased significantly from Day 15 to Day 19 in estradiol valerate induced pseudopregnancy. Robertson and King (1974) and Knight et al. (1977) showed a relatively constant level of progesterone from Day 20-30 until about Day 90-100 when it falls. During this time uteroferrin reaches maximal levels between Days 60 and 75. Estrogen starts to rise from low values at about Day 70 reaching high levels

at term. From this, Roberts and Bazer (1980b) suggested that the progesterone/estrogen ratio, being highest between Days 35-75 of gestation and when uteroferrin production is maximal, may be the mechanism by which the porcine uterus is modulated in its response to progesterone. This mechanism is similar to that proposed for uteroglobin control.

Schlosnagle et al. (1974) further characterized uteroferrin and found that it contined one atom of iron per 32,000 molecular weight polypeptide with an absorption maximum at 545 nm. In addition, it had acid phosphatase activity (pH optimum at 4.9) preferring p-nitrophenyl phosphate as substrate, but also hydrolyzing ATP and sodium pyrophosphate. The native phosphatase activity is quite low but Schlosnagle et al. (1974) showed that in the presence of reducing agents, β-mercaptoethanol or ascorbate, the specific activity was readily increased while there was also a shift in the absorption spectrum to a maximum at 508 nm giving a pink form of the protein. This protein resembles an acid phosphatase purified from beef spleen that is also a strongly basic glycoprotein having a similar absorption spectrum. Additionally, the spleen enzyme is activated by β-mercaptoethanol and ascorbate, and after activation shows a shift in absorption to a shorter wave length. Campbell and Zerner (1973) reported that the beef spleen enzyme contains one atom of iron per molecule.

Schlosnagle et al. (1976) indicated a variety of reagents that cleave disulfide bonds increased the specific activity of the acid phosphatase, but following maximum activation there was a gradual decrease in enzyme activity and color accompanied by a loss of ferrous iron. Apo-uteroferrin (iron-free) could be easily prepared, and

Schlosnagle et al. (1976) clearly showed that Fe^{3+} (ferric) restored the phosphatase activity and color. Also, Cu^{2+}, but not Mn^{2+}, Mg^{2+}, Co^{2+}, or Cr^{2+}, reactivated acid phosphatase activity, but to only 30-50% of the activity of the iron reconstituted protein. Ferrous (Fe^{2+}) iron was unable to restore activity of the enzyme.

Campbell et al. (1978), comparing beef spleen acid phosphatase and the porcine uterine acid phosphatase, revised the molecular weight of the beef spleen enzyme higher and indicated that there were two atoms of iron per molecule of protein. Further, they indicated that the molecular weight of uteroferrin was 40,000, not 32,000 proposed by Chen et al. (1973), and that the uterine protein contained two atoms of iron per molecule of enzyme. More recent data by Basha et al. (1980) and Brumbaugh et al. (1979), however, suggest a molecular weight of uteroferrin around 35,000.

Dionysius et al. (1979) further proposed that one of the two iron atoms per molecule is more easily removed by reduction and that Fe^{2+} is present on the active enzyme rather than Fe^{3+}. Keough et al. (1980) reported that a "one-iron" apoenzyme (lacking enzyme activity) can be prepared and that the acid phosphatase activity was restored with Zn^{2+} ions, however, only Fe^{2+} and Fe^{3+} can restore activity to the iron-free apoenzyme. In addition, Zn^{2+} and Fe^{3+} can restore activity to the iron-free uterine protein but will not give acid phosphatase activity. Other metal ions tested with the "one-iron" and "iron-free" apoenzyme for binding and restoration of enzyme activity (Co^{2+}, Cu^{2+}, Mn^{2+}, and Al^{3+}) gave none. Antanaitis et al. (1980) determined that the iron content is consistent with the presence of one iron atom rather than two per molecule. Further work by Grey, Klimberg and Roberts (unpublished) has

shown that Cu^{2+} can replace iron on uteroferrin, confirming Schlosnagle et al. (1976), and that this yields an active phosphatase with differing pH optimum (5.5 vs 4.9) and absorption characteristics.

The role of uteroferrin, secreted into the reproductive tract during the estrous cycle, is not known but it may serve several functions. One is that it may act as a bacteriostatic agent, similar to that proposed for lactoferrin, by sequestering iron. Since uteroferrin is not found in uterine flushings or allantoic fluid as the apoprotein, this would seem unlikely. Second, it may serve to provide a catalytic function and perform as an acid phosphatase. However, no native substrate has been discovered that uteroferrin prefers. Instead, the low pH optimum of 4.9 with its favored substrate, p-nitrophenol phosphate, its high K_m, and its low activity would argue against this proposal. Third, Roberts and Bazer (1980b) propose that the protein may serve to transport iron from the mother to the fetus. Support for this suggestion is provided by its iron binding property (Schlosnagle et al., 1974), uptake via the areolae on the chorion (Chen et al., 1975), the large amount present in allantoic fluid (Bazer et al., 1975), and its calculated synthetic rate of at least 1 gm/day from Day 60 and 75 explants of pregnant endometrium (Basha et al., 1979). Additionally, Palludan et al. (1969) demonstrated the localization of ^{59}Fe, injected into the maternal circulation, in uterine endometrial glands and placental areolae with subsequent localization in the fetus.

If indeed uteroferrin is an iron transport protein (and iron storage protein), it shares this property with several other major proteins found widely dispersed in biological systems. It remains to

be shown experimentally that iron is transported by this protein and turned over (donated to the fetus) and the mechanism explored if that is the function of uteroferrin.

Iron Transport Proteins: General Properties

Transferrin

Transferrin, sometimes referred to as serotransferrin or siderophilin, is a major constituent of serum whose role is iron-binding and transport. The transferrins are glycoproteins having molecular weights in the range of 75,000-81,000, and they consist of a single polypeptide chain with two identical covalently bound carbohydrate groups. This protein is capable of preferentially binding two iron (ferric) ions tightly, but reversibly at the two similar but not identical binding sites.

In addition, transferrin can bind a wide variety of other di- and trivalent metal ions, some physiologically important, e.g., Cu^{2+} and Zn^{2+}, but it does not bind ferrous iron. For each ferric ion bound at a specific site of transferrin, one bicarbonate or carbonate anion is concomitantly bound. The two sites of binding are not equivalent and are independent of each other.

The mechanism of iron-binding to transferrin is not well understood, but binding involves anion binding, at least two tyrosyl residues, at least one histidyl residue, and possibly a lysine or arginine residue. The mechanism of iron release has been examined by many, and their results suggest at least four mechanisms either alone or in some combination that are involved in release: protonation of the protein's iron-binding ligands, chelation by ligand exchange, reduction, and attack on the anion.

The N-terminal and C-terminal fragments of human and bovine transferrin have been produced for studies of iron-binding and its release. Further, this will allow studies to evaluate the proposal that this protein has arisen by gene duplication with subsequent differentiation of the N-terminal and C-terminal regions.

The most important role of transferrin is in transport of iron between the sites of absorption, storage, and utilization. Interaction with the reticulocyte system is well documented, and its role as an acceptor in the liver and intestine is noted. Further, it may have a protective role as a bacteriostatic agent by restricting the availability of iron (Aisen and Brown, 1975; Chasteen, 1977; Aisen and Listowsky, 1980).

Lactoferrin

Lactoferrin, also called the red milk protein, and lactotransferrin, is found primarily in external secretions such as milk, tears, nasal and bronchial fluid, vaginal and intestinal fluids, hepatic bile, pancreatic juice, and seminal fluid. This protein belongs to the transferrin class of two-sited, single-chain metal-binding proteins. The chemical and physical properties described for transferrin (see Aisen and Listowsky, 1980; Chasteen, 1977), in general, apply to lactoferrin although it has not been studied to the extent of the serum protein.

Lactoferrin is capable of binding two iron (ferric) ions or Cu^{2+} with incorporation of two molecules of bicarbonate or carbonate. The absorption spectra, molecular weight, and amino acid compositions are similar to transferrin although the amino acids are dissimilar enough to prevent a common antigenicity. Lactoferrin is a basic

glycoprotein having a pI > 8, while transferrin has a pI around 5-6. The iron-lactoferrin complex is more stable at low pH (<5) than is the transferrin complex and the binding constant for iron is higher. In addition, lactoferrin contains a fucose residue on each of its oligosaccharide side chains and lacks sialic acid (Prieels et al., 1978). Fucose is responsible for rapid clearance when it is introduced into the circulation.

The mechanism of iron release is not well studied but some evidence shows protein (Lf) uptake by macrophages via specific receptors, followed by iron accumulation in ferritin and lactoferrin degradation (van Snick et al., 1977). Evidence is provided (see Brock, 1980) that describes the apparent susceptibility of lactoferrin to proteases and indicates that the apo form (iron-free) is extremely labile, a characteristic shared with transferrin.

The biological function of lactoferrin in vivo has not been established. Its role as a bacteriostatic or bacteriocidal agent has been documented in vitro and it undoubtedly is due to chelating available iron. Additionally, its role in iron absorption in the gut has been recently examined by van Vugt et al. (1975) and speculated upon. Brock (1980) proposed this to be a major function of lactoferrin.

Ovotransferrin

Ovotransferrin, often called conalbumin, is synthesized in the tubular gland cell of the hen (or stimulated chick) and secreted into the oviduct, a major secreting organ in the hen, becoming a component of egg white. It represents about 50% of the total protein synthesized and is under steroid control. This protein also belongs to the transferrin class of proteins that are two-sited, have a single polypeptide

chain, and are metal-binding. It can bind up to two iron (ferric) ions, requiring the binding of a bicarbonate or carbonate anion for each iron. Generally, the properties ascribed to transferrin hold true for ovotransferrin (Aisen and Listowsky, 1980; Chasteen, 1977).

Various criteria including immunoelectrophoresis, iron-binding properties, amino acid compositions and peptide patterns indicate that the protein components of transferrin and ovotransferrin are identical (Williams, 1962; Gafni and Steinberg, 1974). In fact, the proteins appear to differ only in carbohydrate constituents (Graham and Williams, 1975). Further, it appears that the genes for chick or hen transferrin and ovotransferrin are identical, differing only by their control mechanisms. Ovotransferrin genes (oviduct) are expressed apparently in response to estrogen and progesterone while transferrin genes (liver) are only slightly responsive to estrogen which may not be the primary response (Lee et al., 1978).

The function of ovotransferrin is not known, but many in vitro studies suggest a role as a bacteriostatic agent functioning by sequestering free or available iron. An additional role is that of delivery of iron to the chick embryo red cell (Williams and Woodworth, 1973).

Iron Status During Prenatal and Early Postnatal Development in the Pig

Iron deficiency in the neonate is a major concern of the pig producer. This is a problem man-made because of selection for rapid growing animals and a trend towards large indoor cement-floored environments which greatly restricts the availability of iron (from soil) to the animals. The neonate of the cow, horse, and sheep possess

comparatively large iron stores at birth and are not dependent upon nursing as an iron source. The neonate of the pig is an exception since it does not build large iron stores before birth and, not possessing them at birth, is therefore susceptible to iron deficiency.

To understand further why the pig has low body iron stores, a study of placental iron transfer from mother to fetus is necessary.

The domestic pig, like the horse, whale, and dolphin, has, according to Grosser's classifications, an epitheliochorial placenta. This type is believed to be the "strongest barrier" to transport of materials from mother to fetus, having three layers of maternal tissue and three layers of fetal tissue between the respective blood supplies. In the pig, the mode of nutrient transfer to the fetus is via the embryotropic route, by secretions from the uterine glands. According to Palludan et al. (1969), conditions for direct placental exchange of iron improve as fetal life progresses and the distance between maternal and fetal capillaries becomes reduced to a few microns. This is due to capillary invasion between cells of the uterine epithelium and chorion.

Studying numerous mammals in late gestation, Seal et al. (1972) showed that all placental types, except the hemochorial, exhibited less than 0.1% transfer of a maternal ^{59}Fe injection to the fetus after 24 hours. Hoskins and Hansard (1964) have shown approximately 0.5% transfer of injected iron after 48-72 hours. By contrast, Seal et al. (1972) showed in those mammals having the hemochorial type placenta (with no maternal tissue layers, but three fetal tissue layers), an ^{59}Fe transfer of 6-30% to the fetus within 3 hours after the maternal injection and as much as 48% after 72 hours. Thus, the iron pools of the pig fetus only slowly receive iron from the mother suggesting a circuitous mode of transfer.

Palluden et al. (1969), in another study, examined placental iron transport in pregnant gilts between Days 84 and 90 of gestation by an injection of ^{59}Fe into the maternal circulation. Fetuses were measured at different times after the injection and tissues and fluids examined for radioactivity. The ^{59}Fe was detected in fetal plasma in as little time as 3 hours with a rapid increase in the placental iron pool. Only after larger periods of time did radioactivity accumulate in other tissues, e.g., liver, bone, spleen, and kidneys.

To study the mechanism of iron transfer, Palluden et al. (1969) injected ^{59}Fe-labelled plasma into pregnant gilts and then removed the two uterine horns at different times after the injection and prepared them for histological and autoradiographic examination. Palludan et al. concluded that the uterine epithelium, secretions of the uterine glands, and the uterine secretions at the site of the areolae were highly radioactive based on the high density of radioactive granules at these sites. In another experiment, Palludan et al. (1969) removed placentae from ^{59}Fe-injected pregnant animals after a few hours and punched out large numbers of areolae and interareolar tissue to be counted. The major portion of radioactivity was associated with the areolar tissue. Thus, Palludan suggested that the route of maternal to fetal transport of iron is via the uterine glandular regions and the uterine milk with uptake at the areolae, i.e., via the embryotropic route. This is consistent with Brambell's proposition of direct transfer of nutrients from uterine milk at the areolae to the chorioallantois. In addition, Chen et al. (1975) showed specific transport of uteroferrin in this manner by fluorescent antibody techniques.

In order to prevent iron deficiency anemia in the newborn animal, and to augment the small iron stores of the fetus before birth, various

workers have attempted iron treatment of the mother during late gestation. Positive effects were not observed when sows were injected within four weeks of delivery. However, Ducsay (1977) noted an increase in fetal iron stores and neonatal hemoglobin from sows treated during mid-gestation. The iron treatment was given when secretion of uteroferrin was reaching maximal levels.

After parturition, the meager iron stores of the piglet are inadequate to maintain the rapid growth of the young, since by six weeks the animal should increase its birthweight by 1000%. Additionally, swine milk and colostrum are relatively poor sources of iron from which the nursing piglet obtains only about 1 mg iron/day while requiring 7 mg/day. Thus, if access to soil, which contains large amounts of iron, is prevented, the most successful therapy is parenteral administration of iron-dextran two to three days after birth. This is sufficient to maintain hemoglobin levels.

CHAPTER 2
PROTEIN DETERMINATION, IRON CONTENT, AND MOLECULAR WEIGHT OF UTEROFERRIN

Introduction

Uteroferrin, whose properties have been described previously, is secreted into the lumen of the porcine uterus under the influence of progesterone (Murray et al., 1972; Chen et al., 1973; Schlosnagle et al., 1974). During pregnancy, it is also found in allantoic fluid (Bazer et al., 1975). Recently, Roberts and Bazer (1980) postulated that its function is to transport iron from the mother to the conceptus and it was suggested that uteroferrin resembled transferrin in many of its properties.

Chen et al. (1973) and Schlosnagel et al. (1974; 1976) studied the properties of uteroferrin and reported the molecular weight to be 32,000. They indicated that the protein contained one atom of iron per molecule. The iron, in the ferric (Fe^{3+}) form, was responsible for the purple color and was required for enzymatic activity.

Recently, Campbell et al. (1978) reported that, in their hands, the molecular weight of the uteroferrin from pig allantoic fluid was about 40,000 rather than 32,000 and that there were two iron atoms per molecule of enzyme. Dionysius et al. (1979) suggested that one of the two proposed iron atoms was more easily removed by reduction with dithionite than the other. Further, they proposed that ferrous rather than ferric iron was present on the enzyme and only this ion could reactivate the apoenzyme. Keough et al. (1980) also suggested two iron

atoms per enzyme and that they were in the ferrous form, but they were able to restore significant enzymatic activity with a small excess of ferric iron.

Clearly a controversy exists as to the valence of the iron, the number of atoms bound and the molecular weight of the protein. In this study some of the properties of uteroferrin with regard to these parameters were examined.

Materials and Methods

Materials

Chelex 100 (200-400 mesh) ion-exchange resin was obtained from Bio-Rad, La Jolla, California, and the dry box from Instruments for Research and Industry, Cheltenham, Pennsylvania. Chemicals used were reagent grade or better.

Iron-Free Water

Deionized water was passed by gravity flow through a 600 ml Buchler funnel (medium), containing the sodium form of Chelex 100 resin, into a five gallon glass container previously cleaned with concentrated hydrochloric acid.

Dry Weight Determination

Small aluminum weigh-boats were prepared, washed in 0.1 N HCl and rinsed in iron-free water. The boats were dried in a drying oven at 100°C for 2 hours, placed in an open desiccator containing desiccant, and then placed in a vacuum oven (75-80°C) overnight with constant vacuum. When the oven was opened, it was flushed with dry N_2 through the inlet port and the desiccator closed. The desiccator was transferred to a flexible plastic glove bag (dry box) in which a Cahn Electrobalance (Model G) had been installed and the boats weighed.

Uteroferrin samples were dialyzed extensively against iron-free water containing Chelex resin. Dialysis tubing was prepared by gently boiling in Chelex-treated water containing EDTA and then rinsing in iron-free water. The protein was centrifuged at 3,000 rpm, 4°C, in an I.E.C. DPR 6000 centrifuge to remove any particulate matter, pipetted into the preweighed weigh-boats, and dried from 2 hours to overnight in the drying oven at about 95°C. The dry weigh-boats were transferred to the desiccator and treated similarly to the tare weight determinations. In several experiments, further additions of uteroferrin were made to the boats and the procedure repeated.

Absorbance at 280 nm

Column effluents of uteroferrin were monitored by absorbance at 280 nm to detect peptide material. Protein concentration was determined by absorbance measurements at 280 nm on a Beckman Model 25 double beam spectrophotometer using a 1 cm quartz cell and subsequently related to the dry weight of the protein and protein concentration as determined colorimetrically by the method of Lowry et al. (1951).

Protein Determination

Protein concentration was determined colorimetrically by the method of Lowry et al. (1951). Standards used were bovine serum albumin (BSA), human serum albumin (HSA), and human transferrin (HTF).

Iron Determination

Iron was measured by the method of Cameron (1965) using bathophenanthroline disulfonate as modified by Campbell and Zerner (1973). All glassware was soaked in concentrated HCl and rinsed a minimum of six times with iron-free water to remove metal contaminants. Reagents were analytical grade.

Reagents were prepared as follows: (1) Stock iron solution (100 µg/ml) was prepared by dissolving 70.2 mg of ferrous ammonium sulfate [$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$] (Fisher ACS Grade) in 100 ml of 0.1 N HCl and working iron standards (0-4 µg) were prepared by dilution with the same diluent; (2) hydroxylamine hydrochloride was prepared by adding 10 gm and diluting to 100 ml with iron-free water; (3) bathophenanthroline sulfonate solution was prepared by dissolving 40 mg in a total volume of 10 ml Chelex-treated water.

Protein, blank, and standards were pipetted and treated with 70% (v/v) perchloric acid (200 µl) for 30 minutes at room temperature. After the $HClO_4$ treatment, hydroxylamine (100 µl) was added and allowed to stand for another 30 minutes. Finally, bathophenanthroline sulfonate (100 µl), pyridine (500 µl), and water were added successively and mixed. The absorbance of the solutions was measured at 536 nm. The concentration of iron in uteroferrin is obtained from a plot of absorbance versus the iron concentration of the standards. The plot was linear over the range 0-4 µg.

Purification of Uteroferrin

Uteroferrin was purified from uterine secretions of pseudopregnant pigs or fresh allantoic fluid as previously described (Schlosnagle et al., 1974; Roberts and Bazer, 1980). Briefly, this involves dialysis of the allantoic fluid overnight (4°C) against Tris-HCl buffer (0.01 M, pH 8.0) which was followed by addition of a slurry of carboxymethyl cellulose (CMC) (100 ml settled CMC/liter allantoic fluid) at high pH (0.01 M Tris-HCl, pH 8.0). This was stirred for about 15 min and then packed in a small column (various sizes used). The CMC was washed with the same buffer and then eluted in one step by high salt (0.01 M Tris-HCl, 1.0 M

NaCl, pH 8.0). The protein was dialyzed, concentrated by treatment with PEG, applied to a Sephadex G-100 column (2.5 x 90 cm) previously equilabrated, and was eluted with 0.01 M Tris-HCl, 0.33 M NaCl, pH 8.0. The uteroferrin peak (which eluted between 200 ml and 350 ml) was pooled, dialyzed against Tris-HCl buffer (0.01 M, pH 8) and applied to a column of CM-cellulose to remove the last traces of impurity. Elution was performed using a linear salt gradient (0-0.5 M NaCl) in the loading buffer. Uteroferrin elutes as a symmetrical peak between 0.15 M and 0.2 M NaCl.

SDS-Polyacrylamide Gel Electrophoresis

Electrophoresis in tube gels (0.5 x 7.5 cm) containing SDS was performed in a Bio-Rad electrophoresis cell using the procedure of Laemmli (1970) and Cleveland et al. (1977) employing 15% (w/v) polyacrylamide gels.

The running gel was prepared by mixing the following solutions: 10 ml acrylamide stock solution (30:0.8 by weight acrylamide:bis acrylamide), 5 ml running gel buffer (Tris-HCl containing TEMED, pH 8.8-9.0), 100 µl 20% (w/v) SDS, 5 µl TEMED, and 5 ml ammonium persulfate (0.28 gm to 100 ml water, prepared fresh). After gentle swirling, 1 ml was pipetted into each tube and chemical polymerization was complete by 20 minutes.

The stacking gel was prepared by mixing the following in subdued light: 2.4 ml acrylamide stock solution (30:0.8 by weight acrylamide: bis acrylamide), 2 ml stacking gel buffer (Tris-HCl containing TEMED, pH 6.6-6.8), 80 µl 20% (w/v) SDS, 9.6 ml water, and 2 ml riboflavin mix (4 gm riboflavin to 100 ml water). After gentle swirling, 0.2 ml is pipetted on top of the running gel and the tubes placed under a UV lamp and after 30 min photopolymerization is complete.

Samples and standards were solubilized by the addition of SDS and β-mercaptoethanol to the final concentrations of 2% (w/v) and 10% (v/v), respectively, and boiling for 3 minutes. An amount of sucrose equivalent to about 10% (w/v) of the total volume was added along with 2 µl bromphenol blue. Samples and standards were loaded (25-100 µl), stacked at 1 mA/tube and run at 2 mA/tube. Electrophoresis chamber buffer was composed of the following: 3 gm Tris, 14.4 gm glycine, and 1 gm SDS dissolved in 1 liter, pH 8.3.

After electrophoresis, the proteins were fixed in acetic acid: ethanol:water (7:40:53, v/v) and stained with 0.125% (w/v) Coomassie brilliant blue R-250. Gels were destained in acetic acid:ethanol:water (7:10:83, v/v).

Standards used were bovine serum albumin, ovalbumin, aldolase, pepsin, chymotrypsinogen A, cytochrome C, and β-lactoglobulin.

Results

Molecular Weight Determinations

Using 15% (w/v) polyacrylamide disc gel electrophoresis in the presence of SDS, uteroferrin was found as a single polypeptide chain. The estimated molecular weight from plots of log molecular weight versus mobility was found to be 34,000 in two experiments.

Relationship Between Dry Weight, Optical Properties, and Protein Concentration Determined by the Method of Lowry et al. (1951)

Table 2-I summarizes the relationship obtained between protein dry weight, absorbance at 280 nm and 545 nm and protein as determined by the method of Lowry et al. (1951). With the latter method, two protein standards, human serum albumin (HSA) and human transferrin were employed. Transferrin has an amino acid composition resembling uteroferrin and it gives slightly higher values for protein concentration of uteroferrin

Table 2-I

Relationship Between Uteroferrin Protein Dry Weight, Optical Properties, Colorimetric Determined Protein, Iron Content and the Calculated Minimum Molecular Weight[1]

Uteroferrin Preparation	Protein Determination: Dry Weight (mg/ml ± S.D.)	A_{280} (cm^{-1} ± S.D.)	Colorimetric (mg/ml ± S.D.)	A_{280}/A_{545}
A	1.85 ± 0.23[2]	2.06	1.31 ± 0.05[3] 1.42 ± 0.03[4]	13.73
B	2.79 ± 0.06[5]	3.31 ± .11	2.55 ± 0.17[3] 2.57 ± 0.18[4] 2.60 ± 0.21[6]	13.23
C	4.97 ± 0.02[8]	5.61 ± .19	----------	13.42
D	4.61 ± 0.06[9]	5.47	3.99 ± 0.21[6]	14.02
E	3.14[10]	----	2.64 ± 0.05[4]	-----
F	2.69[10] 2.62[12]	3.02	2.26 ± 0.11[4]	13.74

(1) Values reported as Mean ± S.D.
(2) One sample determined by four different weighings
(3) Human serum albumin used as standard
(4) Human serum transferrin used as standard
(5) Three samples each determined in triplicate
(6) Bovine serum albumin used as standard
(7) Based on colorimetric determination of protein concentration
(8) Two samples each determined in duplicate
(9) Two samples each determined once
(10) Calculated dry weight using the colorimetric protein determination and the dry weight/colorimetric ratio (1.186)
(11) Determined using the dry weight calculated in (10)
(12) Calculated dry weight using the A_{280} protein determination and the dry weight/A_{280} ratio (.867)

Dry Weight/ Colorimetric	Dry Weight/ A_{280}	Iron Content (Mean % by weight ± S.D.)	Molecular Weight(min) (Mean ± S.D.)
1.41(3) 1.30(4)	0.897	0.149 ± 0.015	37,650 ± 3830
1.09(3) 1.09(4) 1.07(6)	0.843	0.169 ± 0.006 0.181 ± 0.006(7) 0.166 ± 0.014	33,180 ± 1180 30,920 ± 1100(7) 33,760 ± 2910
-----	0.886	0.196 ± 0.014	28,600 ± 2350
1.16(6)	0.843	0.191 ± 0.004	29,130 ± 500
-----	-----	0.173 ± 0.003(7) 0.145 ± 0.002(11)	32,230 ± 470(7) 38,460 ± 560(11)
-----	-----	0.178 ± 0.009(7) 0.151 ± 0.008(11)	31,460 ± 1680(7) 37,050 ± 1980(11)

than does HSA. It is not clear why the colorimetric values for sample A were higher in relation to protein dry weight than the other samples.

Freshly purified Uf usually has a ratio of absorbance at 280 nm to 545 nm of around 13.2 (Roberts and Bazer, 1980b). Ratios higher than this indicate either the presence of impurities or, more usually, loss of iron and denaturation of protein. Of the samples used in this study only D had a ratio greater than 14.0. The ratio of dry weight to absorbance of samples A through D averaged 0.867. This value can therefore be used directly to determine the quantity of Uf in any aqueous sample of the protein and was used to calculate the dry weight in sample F (0.867 x A_{280} measurement = Dry Weight).

Assuming a molecular weight of 35,500 (Roberts and Bazer, 1980b) and a ratio of 13.2 for the A_{280}/A_{545} of the best preparation of uteroferrin, the molar extinction coefficient at 545 nm can be calculated to be approximately $3.1 \times 10^3\ M^{-1}$.

Iron Content of Uteroferrin

In these studies the iron content of six separate samples of uteroferrin was determined colorimetrically. The values for each individual sample was measured on several replicates. Based on the dry weight of protein determined for samples A-D and calculated for E and F, the iron content averaged 0.167% with a range between 0.145% and 0.196%. On this basis, the minimum molecular weight of uteroferrin can be calculated to be 33,920 with a range from 28,190 to 38,460.

These results are consistent with the presence of one rather than two iron atoms per polypeptide chain.

Release of Iron from Uteroferrin by Dithionite

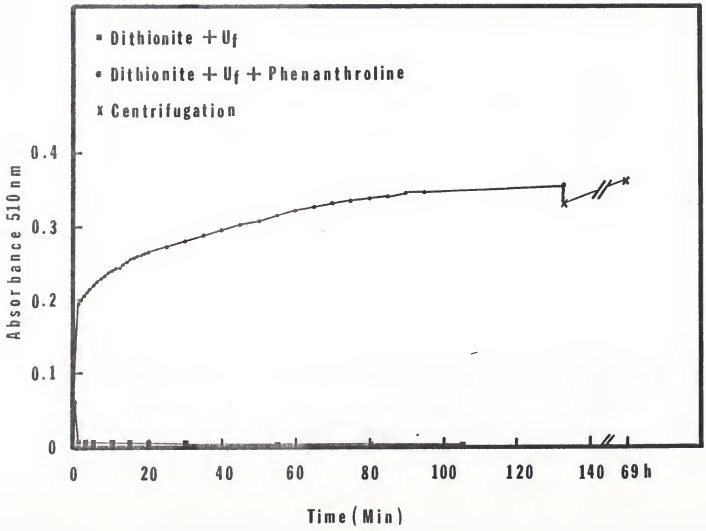
Keough et al. (1980) presented evidence that when uteroferrin was reduced with dithionite (5 mM) and iron release measured colorimetrically, there was an initial rapid loss of one iron atom followed by the slower loss of the second over a period of several hours. However, when the experiment was repeated in this laboratory, it was found that about 60% of the total iron released was lost within 2 min with the remaining iron lost slowly over about 90 minutes. A fine precipitate was observed accompanying this increase in optical density. This was removed at the termination of the reaction and resulted in a slight decrease in the observed optical density. Calculations after centrifugation and a final reading at 69 h gave absorbance readings equivalent to release of 1.23 - 1.3 moles Fe/mole protein (Figure 2-1). In these experiments the reagents and glassware used were not iron-free and the A_{280}/A_{545} ratio was high (14.9).

Discussion

Recently, Campbell et al. (1978) compared the properties of uteroferrin to those of another acid phosphatase from beef spleen. In that paper a number of properties previously established by Chen et al. (1973) and Schlosnagle et al. (1974; 1976) were disputed. The molecular weight according to Campbell et al. was about 40,000 by gel filtration, SDS-gel electrophoresis, and ultracentrifugation. Previously Chen et al. determined the molecular weight to be 32,000 by gel filtration, SDS-gel electrophoresis (using the continuous system of Weber and Osborn), and sedimentation equilibrium centrifugation. Since that time it became apparent that the molecular weight obtained by sedimentation equilibrium centrifugation was low due to the use of an incorrect

Figure 2-1. Iron Loss from Uteroferrin Measured by the Formation of the Iron (II)-1,10-phenanthroline Complex after Reduction by Sodium Dithionite.

Uteroferrin (25 μM) was incubated with sodium dithionite (5 mM) and 1,10-phenanthroline (10 mM) in sodium acetate (0.1 M, pH 4.9). The formation of the iron (II)-1,10-phenanthroline complex was followed at 510 nm. The A_o is 0.060 due to uteroferrin.


▪ Dithionite + Uf
• Dithionite + Uf + Phenanthroline
x Centrifugation
Absorbance 510 nm
0.4
0.3
0.2
0.1
0
0
20
40
60
80
100
120
140
69 h
Time (Min)

partial specific volume (0.64 vs 0.73). Using the revised value for ν a weight average M_r of 35,139 (Buhi et al., 1979) was obtained. Further studies by Basha et al. (1980), using SDS-electrophoresis according to Laemmli (1970), revealed a molecular weight of 37,500 and Brumbaugh et al. (1979), using scanning gel chromatography on Sephadex G-100, indicated a molecular weight of 33,500. Data presented in this study with 15% polyacrylamide gel electrophoresis in the presence of SDS revealed a molecular weight of 34,000. All of these molecular weight determinations are less than those reported by Campbell et al. (1978); on the basis of these results, we suggest that the molecular weight of uteroferrin is around 35,000.

Campbell et al. (1978) and Keough et al. (1980) further suggest that there are two iron atoms per molecule rather than the one proposed by Schlosnagle et al. (1976). Due to this disagreement, the iron content was determined on a number of different preparations of uteroferrin. For this, an accurate estimation of protein concentration by dry weight was necessary since the A_{280} and colorimetric methods are only estimations depending upon the content of aromatic amino acids and particularly tryptophan content. Based on the dry weight measurements and iron analysis, the iron content was found to be 0.167%. Further, the minimum molecular weight for these was shown to be 33,920 which is consistent with the presence of one iron per 30-35,000 dalton peptide. In earlier work, published in abstract form (Buhi et al., 1979), we reported an iron content of 0.175% using atomic absorption spectroscopy which is in good agreement with the colorimetric data. Antanaitus et al. (1980) suggest that the higher the purity of the protein, a low A_{280}/A_{545}, the lower the adventitious iron.

The careful determination of dry weight of four preparations of uteroferrin indicate that an absorption of 1.0 at 280 nm represents a protein concentration of 0.867 mg/ml. On this basis and assuming a molecular weight of 35,500 and a value of 13.2 for the ratio of absorbance of 280 nm to 545 nm, the molar extinction coefficient at 545 nm is $3.10 \times 10^3\ M^{-1}$. This is slightly higher than the value reported by Roberts and Bazer (1980b) who employed a protein concentration not based on dry weight. It is also higher than the extinction coefficient reported by Campbell et al. (1978). Most earlier work was carried out on samples which were probably partially denatured. This is true also for the experiments from Zerner's laboratory who have reported using samples with an A_{280}/A_{545} ratio as high as 15.2.

From this study it is not clear whether iron is in the Fe^{3+} or Fe^{2+} form. Zerner's group (Dionyus et al., 1979; Keough et al., 1980) reported no restoration of enzymatic activity to the apoenzyme by addition of 10^{-3} M Fe^{3+} salts. However, even at concentrations as low as 10^{-5} M, Fe^{3+} is a potent inhibitor of phosphatase activity whereas Fe^{2+} is not (Grey and Roberts, unpublished). Schlosnagle et al. (1976) have shown that phosphatase activity and color are restored when Fe was supplied in only slight molar excess. The Fe^{2+} form, on the other hand, activated only slowly (probably after oxidation to Fe^{3+}). Moreover, Antanaitus et al. (1980) indicated that both pink (reduced and active phosphatase form) and the purple (oxidized and phosphatase inactive form) contained the same valence form of iron (probably low spin Fe^{3+}). The conclusions to be drawn from these results are that uteroferrin consists of a single polypeptide chain ($M_r \sim 35,000$) having one atom of ferric (Fe^{3+}) iron.

CHAPTER 3
IN VITRO STUDIES OF UTEROFERRIN TURNOVER AND IRON LOSS

Introduction

Uterine flushings from nonpregnant female pigs between Days 12-16 of the estrous cycle have a characteristic purplish hue (Murray et al., 1972) due to the presence of a purple-colored, iron-containing glycoprotein (uteroferrin) which has acid phosphatase activity. The same protein is found in the allantoic fluid (A.F.) of pregnant pigs and appears to be transported there across the placenta (Chen et al., 1973; Bazer et al., 1975; Schlosnagle et al., 1976; Roberts et al., 1976). The properties and characteristics of uteroferrin have previously been discussed in Chapters 1 and 2. Roberts and Bazer (1980) suggested that the major and possibly predominant role of uteroferrin is to transport iron from the mother to the fetus. This concept was proposed because of the high rate of UF synthesis in the uterus, its iron content, the amounts that accumulate in allantoic fluid, the sluggish acid phosphatase activity, the low pH optimum, and the apparent lack of a specific native substrate.

A few preliminary observations have suggested that uteroferrin is not passively sequestered in allantoic fluid but may turn over there. In a preliminary study, for example, ^{59}Fe-uteroferrin was incubated *in vitro* in fresh allantoic fluid and a portion of the ^{59}Fe was lost in a time-dependent manner (Roberts, unpublished). Secondly, we noted over a number of years that sterile allantoic fluid samples stored unfrozen prior to fractionation lost their acid phosphatase activity

and provided a poor source of Uf for purification. By contrast Uf alone is stable for many days even when stored at room temperature.

The in vitro experiments discussed in this section were designed to determine a) if uteroferrin is indeed stable in allantoic fluid and b) how the iron might be lost. Previously, it was shown that Fe may be lost from Uf after reduction to Fe(II) (Schlosnagle et al., 1976), thus suggesting a possible mechanism for iron release in vivo. However, other mechanisms might include proteolysis or a direct removal of Fe(III) by a chelator of higher avidity for Fe than Uf. These chelators could conceivably be either iron-binding proteins or low molecular compounds. It was also felt that the experiments might give some insight into the final stages of iron transport to the fetus.

Materials and Methods

Materials

Carrier-free $Na^{125}I$ was obtained from Union Carbide (17 mCi/μgI) and Amersham Corporation (13-17 mCi/μgI). $FeCl_3$ (7-9 mCi/mgFe) was purchased from Amersham Corporation. All inorganic chemicals were reagent grade or better. Porcine albumin and α-fetoprotein were a gift from Dr. R. Stone, Clay Center, Nebraska. Porcine lactoferrin was a gift to Dr. R. M. Roberts from Dr. R. A. Gibbons and Dr. S. N. Dixon.

Animals

Sexually mature crossbred gilts were checked daily for estrus in the presence of intact boars. The animals were bred and this time was designated Day 0. These gilts were divided into groups (2 animals per group) whose pregnancies would be ultimately terminated at either Day 30, 45, 60, 75, 90, or 105. Pregnancy was interrupted by hysterectomy (Bazer et al., 1978). Briefly, the reproductive tract was

exposed by mid-ventral laparotomy and the uterus arranged outside the abdomen. The ovaries, uterus, and part of the cervix were removed and immediately placed in an ice bath. The uterus was dissected open to expose each conceptus, i.e., fetus, placental membranes, and fetal fluids. Allantoic and amniotic fluid samples were collected; the volumes measured and fetuses and tissues weighed. All allantoic fluids were filter sterilized by passage through a .20 μ Nalgene filter.

Acid Phosphatase Assay

Because uteroferrin phosphatase activity is strongly dependent upon its redox state, a standard assay procedure was developed in order to obtain consistent values. To activate the enzyme, 0.1 ml of sample in duplicate was added to 0.4 ml of buffer (0.05 M Tris, pH 7.4 containing 0.1 M β-mercaptoethanol) and mixed using a Vortex mixer. This solution was incubated at 37°C for 20 min, the optimum time for establishment of full phosphatase activity (Schlosnagle et al., 1976). After addition of sodium acetate (0.1 ml, 1 M, pH 4.9) to adjust the pH to that optimal for enzymatic activity, substrate (0.5 ml, 40 mM p-nitrophenyl phosphate) in sodium acetate (0.01 M, pH 4.9) was added. The solution was thoroughly mixed on a Vortex mixer and incubated at 37°C for 10 minutes. The reaction was terminated by addition of sodium hydroxide (1.5 ml, 1 M) and the reaction product assayed by its absorbance at 410 nm. A standard curve of p-nitrophenol in alkali was previously prepared.

Absorbance

Column effluents were monitored by absorbance at 280 nm to detect peptide material and at 545 nm to follow uteroferrin elution, at 463 nm

the absorbance maximum for transferrin and at 410 nm to detect Sóret Bands. The latter was useful as a measure of blood contamination.

Immunoprecipitation

It was necessary to determine the optimal first antibody (sheep antiuteroferrin) concentration so that the precipitation reaction would be at the equivalence point to provide maximum precipitation. The antibody was titrated against Day 60 allantoic fluid which contained uteroferrin of unknown concentration and a trace of ^{125}I-uteroferrin. To accomplish this, 0.1 ml of the first antibody (in duplicate) was employed in the following dilutions: undiluted, 1/5, 1/10, 1/20, 1/40, 1/100, 1/500, 1/1000, 1/2000, 1/4000, 1/6000, 1/8000, 1/10,000, and 1/20,000. These were added to 25 µl of allantoic fluid in sodium barbital (1.0 ml, 0.02 M, pH 7.5) and the mixture incubated for 3 days at 4°C. At this time 0.1 ml of sheep γ-globulin as carrier (400 µg/ml) and 0.2 ml of second antibody (rabbit antisheep γ-globulin, 1/10 dilution of stock antibody) were added and allowed to incubate for a further 2 days at 4°C. The precipitate was removed by centrifugation (2250 x g, 30 min, 4°C) and the supernatant saved for counting. The pellet was resuspended in cold buffer (2.0 ml, 0.02 M sodium barbital, pH 7.5), centrifuged again and the collected precipitate and previous supernatant fraction assayed for ^{125}I content. From these data the percent uteroferrin trapped as an immune complex was calculated. The maximum binding was achieved at a first antibody dilution of about 1/7 (Figure 3-1) with Day 60 allantoic fluid. The immunoprecipitation assay of the incubated allantoic fluid samples was as described above for the titration and the first antibody concentration was 1/7. In an earlier experiment, the assay conditions differed in amounts of sample,

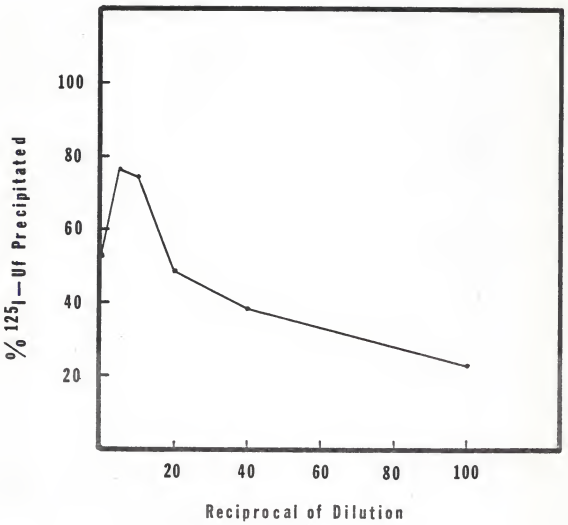


Figure 3-1. Titration of Sheep Anti-Uteroferrin Against a Day 60 Allantoic Fluid Containing ^{125}I-Uteroferrin.

first antibody and second antibody, and the incubation time but the first antibody dilution was essentially the same (i.e. 1/10).

Purification of Uteroferrin

Uteroferrin was purified from fresh allantoic fluid as previously described (Roberts and Bazer, 1980). Briefly, this involves dialysis of the allantoic fluid overnight at 4°C against Tris-HCl buffer (0.01 M, pH 8.0) followed by the addition of a slurry of carboxymethyl cellulose (100 ml settled CMC/liter allantoic fluid) at high pH with the same buffer. This was stirred for about 15 min and then packed in a small column (various sizes used). The ion-exchange resin containing the uteroferrin was washed with buffer and then eluted in one step by high salt (0.01 M Tris-HCl, 0.5 M NaCl, pH 8.0). The protein was dialyzed and applied to a Sephadex G-100 column (2.5 x 90 cm) which was equilibrated and then eluted with a Tris-NaCl buffer (0.01 M, 0.33 M, pH 8.0). The uteroferrin peak (which eluted between 200 ml and 350 ml) was pooled, dialyzed against Tris-HCl buffer (0.01 M, pH 8.0) and applied to a column of carboxymethyl cellulose (of variable sizes) to remove the last traces of impurity. Elution was performed using a linear salt gradient (0-0.5 M NaCl) in the loading buffer. Uteroferrin elutes as a symmetrical peak between about 0.15 M and 0.2 M NaCl.

Apo-Uteroferrin Preparation

Sodium dithionite was added (to a final concentration of 0.1 M) to uteroferrin in sodium acetate (0.1 M, pH 4.9) or Tris-NaCl (0.01 - 0.05 M, 0.15 M, pH 7.5). This solution was held at room temperature for at least 10 min and no longer than 1 hour. This procedure results in loss of the purple-color within 10 min at pH 7.5. Enough of the iron chelator 2, 2'-bipyridine which forms a red-colored complex with

Fe(II) was added to give a ratio of chelator to iron of 3:1 (w/w). The iron-free protein was separated from the iron complex by gel filtration on Sephadex G-25 (1.5 x 20-30 cm). Fractions were monitored at 280 nm and the apo-uteroferrin used immediately for preparation of ^{59}Fe-Uf since the apoprotein readily denatures (Schlosnagle et al., 1976). All glassware was soaked in concentrated HCl and rinsed in Chelex-treated water. All buffers were prepared in iron-free water. The Sephadex gel was prepared by swelling in iron-free water and then gently boiled according to the manufacturer's directions. The Sephadex and all buffers were degassed and N_2 gas bubbled through before use to prevent reoxidation of Fe(II) to Fe(III).

Preparation of ^{59}Fe-Uteroferrin

Up to 0.5 mC $^{59}FeCl_3$ was added to freshly prepared apo-uteroferrin and allowed to incubate 3-18 h at room temperature. The ^{59}Fe-uteroferrin was separated from free ^{59}Fe by gel filtration on Sephadex G-25 (1.5 x 20-30 cm) and fractions monitored by counting radioactivity on a γ-counter. Labelled uteroferrin was concentrated by allowing it to bind to a column of carboxymethyl cellulose (1 x 1.5 cm). It was eluted as a concentrated band with 0.5 M NaCl in Tris-HCl (0.01 M, pH 8.0).

Labelling of Uteroferrin with ^{125}I

Lactoperoxidase Techniques. One milligram of lactoperoxidase (Sigma Chemical Company or Calbiochem) was dissolved in 1.5 ml Tris-buffered saline (0.05 M, 0.15 M, pH 7.4) to which uteroferrin (0.7-5 mg) in various buffers (0.1-1.0 ml) was added. Carrier-free Na^{125}I (1-2 mC) in phosphate buffer (0.5 M, pH 7.5) was first added followed by 0.06% H_2O_2 (10 µl) every minute for 10-20 minutes. Separation of labelled

protein from free-iodine was accomplished by gel filtration, first on Sephadex G-50 (1.5 x 25 cm) followed by loading the pooled void volume peak of ^{125}I-uteroferrin on Sephadex G-100 (1.5 x 65 cm). The 1 ml column fractions were monitored by following the radioactivity on a γ-counter.

Iodo-Gen Technique. The iodination was performed essentially according to the method of Markwell and Fox (1978) and Markwell (Pierce Chemical Company Bulletin). Acid washed 13 x 100 mm glass tubes were gently coated with 10 µg or 100 µg Iodo-Gen (1,3,4,6-tetrachloro 3α, 6α-diphenylglycoluril) (Pierce Chemical Company) by evaporation of chloroform. Uteroferrin (0.05 - 1 mg) in 1 ml of buffer (0.02 M sodium barbital, 0.4 M NaCl, pH 7.5) was added to the Iodo-Gen coated tube previously rinsed in the buffer. Carrier-free Na^{125}I (0.5 - 1 mC) was added and the tube shaken gently for a few seconds every minute for about 10-15 minutes. The ^{125}I-Uf was separated from unreacted ^{125}I by gel filtration first on Sephadex G-50 (1.5 x 25 cm) and then the pooled void volume peak, containing the ^{125}I-Uf, was further purified on Sephadex G-100 (1.5 x 65 cm) as before.

Labelling of Other Proteins with ^{125}I

The iodinations were performed essentially as for uteroferrin using the methods of Markwell for Iodo-Gen. Porcine transferrin (0.4 - 1.1 mg) in 1 ml buffer (usually 0.02 M sodium barbital, 0.4 M NaCl, pH 7.5) was added to the Iodo-Gen coated tube (100 µg) previously rinsed in the labelling buffer. Carrier-free Na^{125}I (0.5 - 1.5 mCi) was added and the tube shaken gently for a few seconds every minute for 10-15 minutes. The ^{125}I-pTf was separated from unreacted ^{125}I by gel filtration on Sephadex G-50 and G-100 as for uteroferrin.

The 1 ml mixture of porcine α-fetoprotein and albumin (1 mg/ml) in solubilization buffer (see below) was added to the Iodo-Gen coated tube (100 µg) previously rinsed in the buffer. Carrier-free $Na^{125}I$ (1 mCi) was added and shaken as before for 10 minutes. The iodinated proteins were separated from unreacted ^{125}I by gel filtration on Sephadex G-50 as before.

SDS-Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was performed in tube gels (0.5 x 7.5 cm) using a standard cell. Separation of small proteolytically cleaved iodinated peptides from uteroferrin during incubation in allantoic fluid was performed in 15% (w/v) acrylamide gels according to the procedure of Laemmli (1970) and Cleveland et al. (1977).

The running gel was prepared by mixing the following solutions: 10 ml acrylamide solution (30:0.8 w/w acrylamide to bisacrylamide), 5 ml running gel buffer (Tris-HCl containing 0.12 ml TEMED/100 ml, pH 8.8 - 9.0), 0.1 ml 20% (w/v) SDS, 5 µl TEMED and 5 µl ammonium persulfate (0.28 gm to 100 ml water, prepared first before use). After gentle swirling, 1 ml of the above solution was pipetted into each tube and polymerization was completed in 20 min at room temperature.

The stacking gel was prepared by mixing the following solutions in subdued light: 2.4 ml acrylamide stock solution (30:0.8 (w/w) acrylamide to bisacrylamide), 2 ml stacking gel buffer (Tris-HCl containing 0.46 ml TEMED/100 ml, pH 6.6 - 6.8), 0.08 ml 20% (w/v) SDS, 9.6 ml water and 2 ml riboflavin mix (4 gm riboflavin in 100 ml water). After gentle mixing, 0.2 ml was pipetted onto the top of the running gel and the tubes placed under a fluorescent lamp. Polymerization was completed within 30 minutes.

Samples and standards were solubilized by boiling in 2% (w/v) SDS and 10% (v/v) β-mercaptoethanol for three minutes. An amount of sucrose equivalent to about 10% of the volume was then added, plus 2 µl bromphenol blue. About 0.05 ml of sample was loaded onto the gel and the samples were allowed to stack at 1 mA/tube for about 1 hour. The current was then increased to 2 mA/tube. The electrophoresis chamber buffer consisted of the following: 3 g Tris-HCl, 14.4 g glycine, and 1 g SDS in 1 liter of water, pH 8.3.

After electrophoresis the gels were removed from the tubes and immediately stored at -70°C. They were later removed from the freezer and sliced into 1 mm slices whose radioactive content was determined. This allowed the location of labelled proteins to be compared with standards along the length of the gel.

Two-Dimensional Electrophoresis

This procedure is a modification by Horst and Roberts (1979) of the original method of O'Farrell (1975).

Isoelectric Focusing (IEF). The IEF gel (0.3 x 11 cm) was prepared by mixing the following solutions: 1.33 ml acrylamide stock solution (31.96% (w/v) acrylamide, 5.64% (w/v) N, N'diallyl tartardiamide), 5.5 g urea, 2 ml 10% (w/v) Nonidet P-40, 0.5 ml ampholyte mixture (0.25 ml pH 3.5-10 Ampholines, 0.18 ml pH 5-7 Ampholines, and 0.08 ml pH 9-11 Ampholines), 0.3 ml riboflavin TEMED mix (4 mg riboflavin, 0.8 ml TEMED in 100 ml water), and 10 µl of ammonium persulfate (0.12 g/ml) in a total volume of 10 ml. Protein was dissolved in solubilization buffer (2.75 g urea, 0.1 ml 10% (w/v) Nonidet P-40, 25 mg DTT, and volume taken to 5 ml with 5 mM K_2CO_3) to 4 mg/ml and up to 150 µl applied to each gel and focused (75V for 15 min; 150V

for 1 h, 300V for 15 h; 450V for 1 h) for a total of 16 to 18 h towards the anode. A blank gel without any sample was occassionally included for subsequent determination of the pH gradient. At the end of the run, the gels were removed from the tubes and equilibrated for 15 min in buffer containing 0.065 M Tris-HCl, pH 6.9, 1% (w/v) SDS and 1% (v/v) β-mercaptoethanol. After equilibration, the gels were drained and either subjected to electrophoresis in the second dimension directly or stored frozen at -70°C.

SDS Gel Electrophoresis. The equilibrated IEF gels were subjected to electrophoresis in the second dimension in 10% (w/v) polyacrylamide slab gels (11 x 0.15 x 11 cm) prepared by following the procedure of Laemmli (1970). The IEF gel was sealed onto the top of the slab gel with a solution of 1% (w/v) agarose, containing 0.065 M Tris-HCl, pH 6.9, 1% (w/v) SDS, and 1% (v/v) β-mercaptoethanol. After electrophoresis, the proteins were fixed in acetic acid:ethanol:water (7:40:53, v/v) with several changes and stained with 0.125% (w/v) Coomassie brilliant blue R-250 for about two hours. The slab gels were destained in acetic acid:ethanol:water (7:10:83, v/v).

Nonequilibrium pH Gradient Electrophoresis (NEPHGE). Proteins of high isoelectric point (basic proteins) were subjected to electrophoresis in the first dimension in a mixture similar to that described above for isoelectric focusing except that the ampholine mixture was of a different composition. The method is based on that of O'Farrell et al. (1977). The 0.5 ml ampholyte mixture used contained 0.35 ml pH 9 - 11 Ampholines, 0.1 ml pH 8 - 9.5 Ampholines, and 0.05 ml pH 6 - 8 Ampholines. Protein migration was toward the cathode at 75V for 15 min and 400V for 3.5 hours. Under these conditions the

proteins do not reach their isoelectric points within the gel. Instead they migrate along a gradient of increasing pH. The gels were removed after this, equilibrated, and subjected to SDS-slab gel electrophoresis in 10% (w/v) acrylamide gels as described above. It should be emphasized that the final positions of the polypeptides in NEPHGE are strongly influenced by the volume of the sample applied and the time of electrophoresis. We routinely used sample volumes of 0.1 ml or 0.2 ml and a running time of 3.5 hours.

Uteroferrin Radioimmunoassay

The concentration of uteroferrin in porcine allantoic fluid, fetal plasma and maternal serum was determined with a double antibody radioimmunoassay system. First antibody was prepared according to Chen et al. (1973) by injection of an emulsion of uteroferrin and Freund's complete adjuvant (Difco Labs, Detroit, Michigan) intradermally on the shoulder, rear leg, and abdominal regions of a female lamb. The second antibody, originally purchased from Antibodies, Inc., Davis, California, was also generated in rabbits against sheep γ-globulin. Later, this second antibody, rabbit antisheep γ-globulin, was prepared in our laboratory by injecting intradermally on the shoulder, back and abdominal regions of a male rabbit 1.0 ml of an emulsion containing 0.5 ml sheep γ-globulin (2 mg/ml) (Nutritional Biochemical Company, Cleveland, Ohio) and 0.5 ml Freund's complete adjuvant. Radiolabelled uteroferrin (^{125}I) was prepared as described under ^{125}I labelling. There was no crossreactivity of the first antibody (sheep anti-uteroferrin) with porcine transferrin or porcine lactoferrin as demonstrated by immunodiffusion on Ouchterlony plates. The antibody gave a strong immunoprecipitation line with purified Uf, however. In addition, no

crossreactivity was noted with porcine transferrin in the radioimmunoassay in the concentration 3.3 μg/ml to 3.3 mg/ml.

Standards were prepared fresh for each assay by diluting a stock preparation of nonradioactive uteroferrin to 100 μg/ml with assay buffer containing the following: 0.02 M sodium barbital, pH 8.0, 1% (w/v) BSA, 0.15 M NaCl and 5 mM EDTA. From this stock solution a 10 μg/ml standard was prepared and from which a portion was diluted to 1 μg/ml. The working standards were comprised of the following concentrations (μg/ml): 0.05, 0.075, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5 and 2.0. The first antibody was used in a dilution of about 1/8000 and the second antibody in a dilution of 1/5 to 1/10. Briefly, the assay consisted of the following: sample, standard or control (0.1 ml), 0.1 ml first antibody, 0.1 ml ^{125}I-uteroferrin (10-20,000 cpm), and 0.5 ml assay buffer incubated at 4°C for 3 days. Then 0.1 ml of sheep γ-globulin (400 μg/ml) and 0.1 ml second antibody were added and incubated overnight at 4°C and centrifuged (2250 x g, 4°C, 30 min) to remove the precipitate. This was washed with ice-cold buffer, recentrifuged, the supernatant discarded, and the precipitate counted. Also included were tubes for total counts, maximum binding, and nonspecific binding (NSB). The sensitivity of the assay with respect to differentiating from 0 μg was 0.1 - 0.2 μg/ml with a range from about 0.1 μg to 1.5 μg. Samples of allantoic fluid were diluted with the assay buffer to insure that the detected levels were within the range of the sensitivity of the standard curve. However, the assay can be adapted for more concentrated or more dilute samples. A validation of the radioimmunoassay is reported in Appendix A.

Results

Supporting data concerning the animals used in this study are in Table 3-1. Gilts were hysterectomized at the various stages of pregnancy (Days 30, 45, 60, 75, 90, and 105) and tissues and fetuses and fluids collected for weighing, volume determination and for a number of subsequent experiments. The data are consistent with previously published results on conceptus development in the pig by other investigators (Knight et al., 1977). Fetal wet weight increased throughout the gestation from a mean ($\bar{x} \pm$ SEM) of 1.33 ± .05 g at Day 30 to 913.88 ± 52.67 g at Day 105, while the placental wet weight increases only up to about Day 60, changing little thereafter. The allantoic fluid volume increased rapidly between Days 20 and 30 (Knight et al., 1977) reaching a peak at about Day 30 (221.75 ± 11.12 ml) which seems to be associated with expansion of the chorioallantoic membranes and establishment of contact between the placenta and the entire endometrial surface of the uterus. A second period of accumulation of allantoic fluid begins after Day 45 and reaches a maximum at about Day 60 of pregnancy (312.15 ± 53.41 ml). There is then a decline to Day 105.

Determination of the Concentration of Uteroferrin in Fetal Fluids

Uteroferrin concentration was determined by a double antibody radioimmunoassay in a) allantoic fluid of pregnant gilts from the various days of gestation (30, 45, 60, 75, 90, and 105); b) fetal plasma (cord blood) from fetuses at Days 60, 90, and 105 of gestation; and c) maternal serum. Figure 3-2 shows that the total amount of uteroferrin (mg/sac) in allantoic fluid (mean ± SEM) increased from 0.14 ± .05 mg/sac at Day 30 to 104.17 ± 22.45 mg/sac at Day 60 and 109.97 ± 22.86 mg/sac at Day 75. Thereafter, there was a decrease

Table 3-I

Comparisons of Fetal Wet Weight, Placental Wet Weight, Allantoic Fluid Volume, Allantoic Fluid Protein Concentration, Total Protein/ Allantoic Sac and Number of Live Fetuses/Pregnant Gilt at Various Stages of Gestation(1)

Days of Gestation(2)	Fetal Wet Weight (g)	Placental Wet Weight (g)	Allantoic Fluid (ml)
30	1.33 ± .05	32.40 ± 2.29	221.75 ± 11.12
45	21.29 ± .52	81.89 ± 6.85	87.40 ± 19.4
60	106.60 ± 3.72	207.00 ± 16.24	312.15 ± 53.41
75	328.15 ± 9.74	234.27 ± 16.35	67.07 ± 14.89
90	665.67 ± 17.91	288.93 ± 14.89	84.10 ± 16.48
105	913.88 ± 52.67	245.86 ± 16.85	30.50 ± 5.91

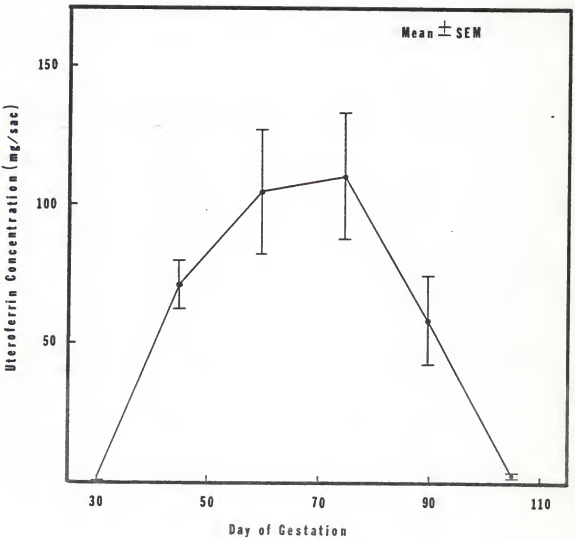
Days of Gestation(2)	Allantoic Fluid Protein (mg/ml)	Total Protein/ Sac (mg)	Live Fetuses/ Gilt
30	0.38 ± .02	83.09 ± 6.36	8
45	2.75 ± .61	208.41 ± 41.47	8
60	1.63 ± .47	361.14 ± 76.21	13
75	4.08 ± .42	289.84 ± 96.92	10
90	2.10 ± .16	196.70 ± 32.59	8.5
105	4.12 ± .70	147.38 ± 30.57	11.5

Gilts at various stages of gestation (Days 30, 45, 60, 75, 90 and 105) were hysterectomized and fetal fluids and tissues collected. The number and the wet weight of the fetuses and placenta were determined. Allantoic fluid volume was measured and protein assayed by the procedure of Lowry et al. (1951).

(1) Mean ± SEM
(2) Two gilts/group except Day 30 which contained one.

Figure 3-2. Total Uteroferrin Content in Allantoic Sacs at Various Stages of Gestation

A double antibody radioimmunoassay was established and the amount of uteroferrin in the allantoic fluid from Days 30, 45, 60, 75, 90 and 105 of gestation determined. Briefly, the assay consisted of the following: 0.1 ml of sample, standard or control, 0.1 ml first antibody, 0.1 ml ^{125}I-Uf (10-20,000 cpm), and 0.5 ml assay buffer all incubated for 72 h at 4°C. To this, 0.1 ml sheep γ-globulin and 0.1 ml second antibody were added and incubated for a further 24 h at 4°C. The resulting precipitate was pelleted by centrifugation, the pellet washed, recentrifuged, and counted. Maximum binding, total counts and nonspecific binding samples were also included.


Mean ± SEM
150
100
50
Uteroferrin Concentration (mg/sac)
30
50
70
90
110
Day of Gestation

toward term with Day 105 samples containing 1.71 ± .40 mg/sac. This implies that at Day 60 (assuming 8-13 fetuses/pregnant animal and a mean content of 104 mg uteroferrin) that from 830 to 1400 mg uteroferrin could be isolated from a single pregnant uterus.

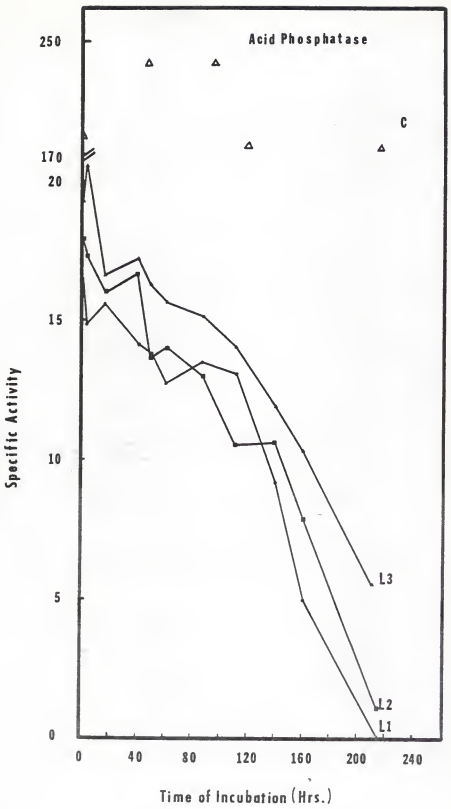
Uteroferrin was undetectable in maternal serum using the radioimmunoassay. By contrast, fetal plasma from fetuses at Day 60 of gestation (n = 22) showed a mean of 2.87 ± .78 μg/ml, although many of these samples (about one-third) had undetectable levels. Fetal plasma from Day 90 (n = 10) and Day 105 (n = 13) fetuses had means of 0.12 ± 0.3 μg/ml and 0.20 ± .02 μg/ml, respectively. Samples earlier than Day 60 were not analyzed due to the small blood volume of the fetuses during the first two months of gestation.

Turnover of Uteroferrin in Allantoic Fluid

Loss of Acid Phosphatase Activity. Uteroferrin accounts for more than 95% of the total acid phosphatase activity in Day 60 allantoic fluid (Bazer et al., 1975). Measuring acid phosphatase activity, therefore, is one way to assay for uteroferrin. Samples of sterile-filtered allantoic fluid from different conceptuses at different days of gestation were incubated at 37°C. Aliquots were removed at designated times and assayed under standard conditions. A typical experiment for Day 60 allantoic fluid is shown in Figure 3-3. In each incubation mixture there was a time-dependent loss of endogenous enzyme activity in allantoic fluid compared with control samples incubated in buffer alone (0.1 M MOPS, 0.15 M NaCl, pH 6.8). However, there was considerable variation both in initial activity and loss of subsequent activity between allantoic fluid samples from different days of pregnancy, i.e., Days 45, 60, 75, 90, and 105, in addition to individual sacs at the

Figure 3-3. Specific Activity of Uteroferrin in Allantoic Fluid After Incubation up to 212 h In Vitro from a Day 60 Pregnant Gilt

Allantoic fluid was collected at various stages of gestation, filter-sterilized and incubated at 37°C in vitro up to 212 h. A control sample in buffer was treated similarly. Samples were removed at various times and frozen at -20°C. All samples were assayed for acid phosphatase activity according to the standard assay procedure. Samples are from pregnant gilt 151R, Day 60, sac L1(●), L2 (■), and L3 (▲) where L refers to the left uterine horn and the number refers to the location of the fetus, one being upper most in uterine horn. The control (△) is pure uteroferrin incubated in MOPS-saline buffer (0.1 M, 0.15 M, pH 6.8). The specific activity is μmole p-NP released/min/mg protein at 37°.

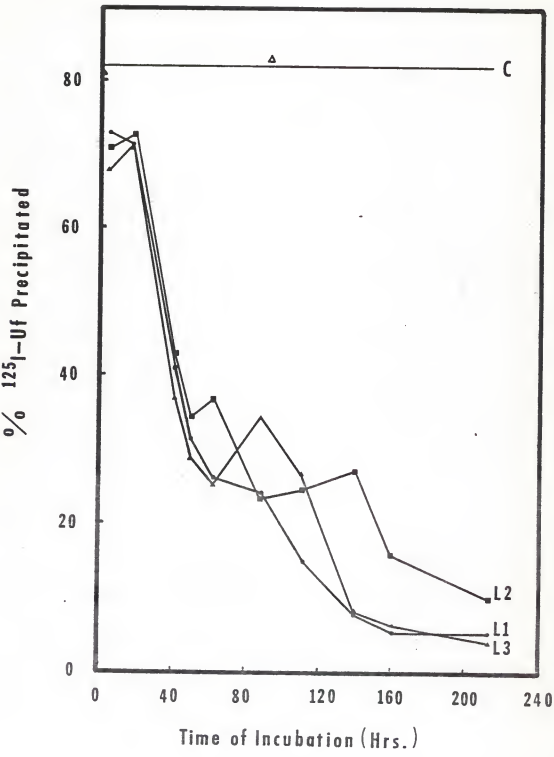

Acid Phosphatase
250
170
20
15
10
5
0
C
L3
L2
L1
0
40
80
120
160
200
240
Specific Activity
Time of Incubation (Hrs.)

same stage of pregnancy (data not shown). From these results we conclude that uteroferrin acid phosphatase activity is unstable in allantoic fluid when it is incubated in vitro at 37°C and that a large amount is lost within days. In general, destruction seemed highest between Days 60 and 90.

Loss of Immunoprecipitable Uteroferrin. The previous experiments indicated that there was a time-dependent loss of acid phosphatase activity from sterile allantoic fluid when it was incubated in vitro. However, the reasons for this inactivation were not clear. In this series of experiments we wanted to determine whether there was a loss of immunoprecipitable Uf polypeptide accompanying disappearance of phosphatase activity. To accomplish this, a small aliquot of ^{125}I-labelled Uf had been added to allantoic fluid before incubation and then the amount of immunoprecipitable uteroferrin was examined. These fluids were obtained from animals at different days of gestation (as above) and each determination was carried out in duplicate at 37°C. Initially, the sheep anti-uteroferrin antibody was titrated against Day 60 allantoic fluid to assure that the immunoprecipitation reaction was in the zone of equivalence. In Figure 3-4 a time-dependent loss of immune precipitable uteroferrin of three different samples of allantoic fluid from separate sacs (L1, L2, and L3) of only the Day 60 pregnant animal is reported. A time-dependent loss of precipitable material was observed, with a half-life of ^{125}I loss occurring in about 40 hours. In all of these experiments we were unable to precipitate more than about 75% of the labelled uteroferrin, including the control in buffer alone. The reasons for this are unclear. The precipitation results, however, are consistent with either a loss of protein or a marked change in the immunological characteristics of the incubated uteroferrin. Note,

Figure 3-4. Percent ^{125}I-Uf Precipitable from Allantoic Fluid after Incubation *In Vitro* from a Day 60 Pregnant Gilt

^{125}I-labelled uteroferrin was added to three sterile-filtered allantoic fluids (L1, L2, and L3) from a single Day 60 pregnant gilt (151R) and incubated at 37°C *in vitro* up to 212 hours. Samples were removed at various times and frozen until analyzed. Using specific anti-uteroferrin antibody, the samples were immunoprecipitated, the pellets collected by centrifugation, washed, recentrifuged and the radioactivity in the pellet determined. Samples are L1 (●), L2 (■), L3 (▲), and control (△).


C
L2
L1
L3
80
60
40
20
0
0
40
80
120
160
200
240
% 125I–Uf Precipitated
Time of Incubation (Hrs.)

however, there was little loss of immunoprecipitable material over the initial 20 h when losses of enzyme activity were beginning, suggesting that the initial loss in enzyme activity may be distinct from the loss of immunological identity.

Proteolytic Cleavage of Uteroferrin. The loss of immunological identity described in the previous section indicated that significant changes in the polypeptide structure of uteroferrin were occurring. The most likely event was that Uf was being degraded proteolytically. To test this, ^{125}I-uteroferrin was added as a tracer to sterile-filtered allantoic fluid containing its own endogenous Uf from different days of gestation and incubated at 37°C (see above). These samples were then analyzed using SDS-electrophoresis in 15% (w/v) polyacrylamide gels to detect small peptide fragments in samples after treatment with SDS and β-mercaptoethanol. Loss of radioactivity from the Uf band and the appearance of radioactive iodine in lower molecular weight bands was followed over a 161 h period and the results shown in Figure 3-5 are for a typical experiment for Day 60 allantoic fluid. Here, a series of smaller molecular weight polypeptides began to appear as ^{125}I-uteroferrin began to decrease. In control incubations in buffer alone, 74-90% of the ^{125}I recovered from the gels appeared to be associated with ^{125}I-Uf (i.e. molecular weight 35,000) throughout the 96 h. The molecular weights of the generated fragments were estimated by polyacrylamide gel electrophoresis in SDS and are listed in Table 3-II. Allantoic fluid samples from different days of gestation were incubated similarly and after 96 h most of the ^{125}I-Uf in Days 45, 60, 75 and 90 samples had been destroyed (data not shown). This destruction occurred in a time-dependent manner while the control (^{125}I-Uf in buffer) was almost

Figure 3-5. Polyacrylamide Gel Electrophoresis of Day 60 Allantoic Fluids Containing ^{125}I-Uf after Incubation *In Vitro* for Various Times

Allantoic fluid from a pregnant gilt (#151R, Day 60, L2) sterile-filtered and containing ^{125}I-Uf was incubated *in vitro* at 37°C. Aliquots were taken at various times and frozen at -20°C. All samples were solubilized by boiling in 2% (w/v) SDS and 10% (v/v) βME for 3 minutes and subjected to electrophoresis in 15% (w/v) polyacrylamide tube gels. The gels, including a 5-mm stacking gel, were sliced using a guillotine of razer blades (1-mm spacers), placed in tubes and the radioactivity of the slices determined. Major fragments are identified in panels 41 h and 62 h.

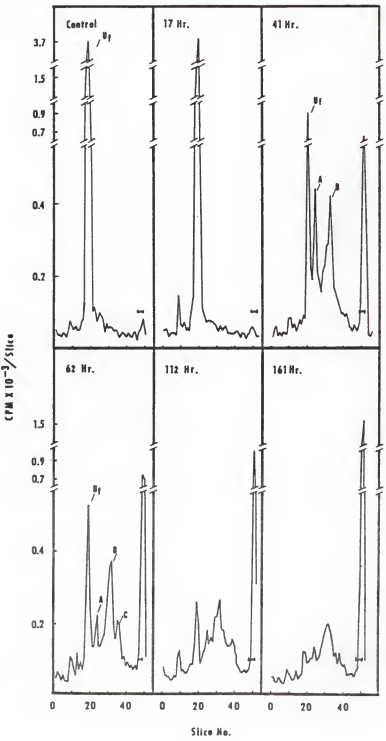

Control
17 Hr.
41 Hr.
62 Hr.
112 Hr.
161 Hr.
Bf
A
B
C
3.7
1.5
0.9
0.7
0.4
0.2
CPM X 10⁻³/Slice
0
20
40
Slice No.

Table 3-II

Molecular Weight of Uteroferrin and Fragments (A, B, and C) Generated by Incubation of ^{125}I-Uf in Day 60 Allantoic Fluid Through 116 h and Estimated by Polyacrylamide Gel Electrophoresis

Material	M_r(1)
Intact Uteroferrin	30,670 ± 420
Fragment A	22,830 ± 105
Fragment B	14,670 ± 210
Fragment C	10,700 ± 580

(1) Mean ± SEM

completely stable. It appears that the destruction did not follow first order kinetics. Rather the initial rate of Day 45, 60, and 75 samples was low since most of the MW 35,000 peak remained at the end of 24 h of the incubation. This was followed, however, by a more rapid phase of proteolytic cleavage. The half-life of uteroferrin in Day 60 allantoic fluid was between 24 and 48 h as determined by these experiments. The possibility that the ^{125}I-Uf is selectively destroyed compared to unlabeled Uf is believed to be unlikely as the iodine labelling was mild, incorporating only about 0.1 - 0.2 moles I/mole protein.

Recent data from Brock et al. (1976) and Williams (1974) have shown that the iron-saturated forms of bovine transferrin and lactoferrin and ovotransferrin are less readily degraded by proteolytic enzymes than are the partially saturated or iron-free forms. We wanted to test the possibility that the iron-free apoprotein was more susceptible to proteolytic cleavage in allantoic fluid than Fe-Uf. To test this, uteroferrin was iodinated and the apoenzyme prepared and added to allantoic fluid and destruction measured as above. Results indicated that the apo-uteroferrin was more rapidly destroyed than the Fe(III) species. This result probably does not represent the true rate of destruction since apo-Uf most likely partially renatures. Clearly, however, removal of iron renders uteroferrin more susceptible to proteolytic cleavage.

Loss of Iron from Uteroferrin. Since significant changes in the polypeptide structure were occurring in uteroferrin when incubated in allantoic fluid, we wanted to know whether iron loss paralleled or preceded the proteolytic destruction of uteroferrin. Iron-^{59} labelled uteroferrin was prepared (Methods) and incubated in filter-sterilized

pooled Day 60 allantoic fluid at 37°C to determine a) how quickly ^{59}Fe was lost and b) whether uteroferrin can donate iron to another protein or chelator in allantoic fluid. The association of ^{59}Fe with uteroferrin was followed by gel chromatography in a column (2.5 x 60 cm) of Sephadex G-100. As seen in Figure 3-6 ^{59}Fe loss from ^{59}Fe-uteroferrin incubated in buffer (0.1 MES, pH 6.5) for up to 96 h was negligible. In allantoic fluid, however, approximately 40% of the ^{59}Fe had been lost in 24 h and had appeared in two additional regions of the column eluate (B and D), eluting between fractions 45 and 60 and between 105 and 130, respectively (Figure 3-7). The former peak (B) corresponded to a protein of molecular weight greater than Uf which eluted in a position nearly identical to serum transferrin. The latter peak corresponded with the salt volume and probably represents either free iron [as Fe(II) or Fe(III)] or iron complexed with a low molecular weight chelator.

With increasing time, ^{59}Fe continued to be lost from uteroferrin at a rate that was approximately first order. The half-life for Fe loss was about 55-60 hours. Interestingly, the low molecular weight material showed no further increase in ^{59}Fe content while the content of protein peak B continued to increase. A second peak (A) containing ^{59}Fe also was evident close to the void volume of the column near the end of the incubation. Its identity is unknown.

Two-Dimensional Polyacrylamide Gel Electrophoresis

These experiments were carried out in order to: a) define the polypeptide composition of allantoic fluid; b) assess the relationship between fetal fluids; and c) confirm that transferrin was present in allantoic fluid at the same time as Uf. Two-dimensional polyacrylamide gel electrophoresis was used to separate proteins on the basis of both

Figure 3-6. Sephadex G-100 Chromatography of ^{59}Fe-Uf After Incubation in Buffer for 48 h and 96 h

^{59}Fe-Uf was incubated in MES buffer (0.1 M, pH 6.5) for 48 h and 96 h. Aliquots were removed, frozen at -20°C until use, thawed, made 0.4 M in NaCl by adding solit NaCl, the radioactivity determined and loaded onto Sephadex G-100 columns (1.5 x 60 cm). The columns were equilibrated and eluted with sodium barbital-sodium chloride buffer (0.02 M, 0.4 M, pH 7.6). Fractions of 1.1 ml were collected and the radioactivity determined.

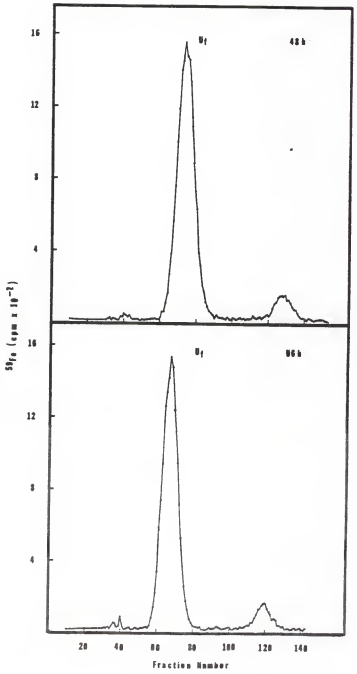
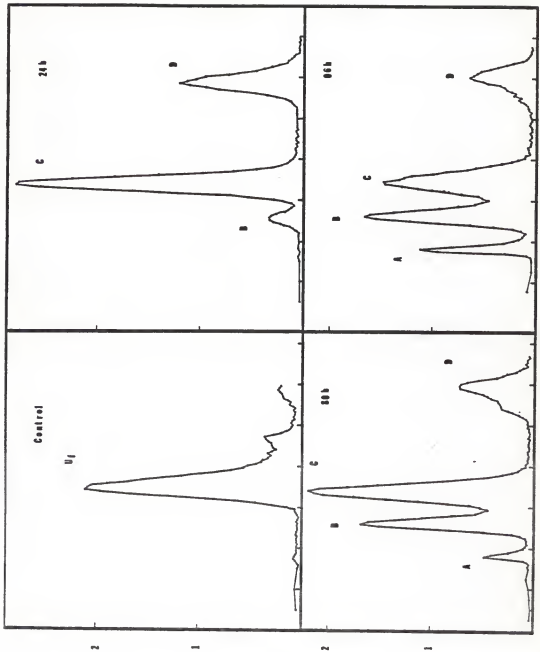

16
12
8
4
U_f
48 h
16
12
8
4
U_f
96 h
SR_{Fe} (cpm x 10^{-2})
20
40
60
80
100
120
140
Fraction Number

Figure 3-7. Sephadex G-100 Chromatography of Pooled Day 60 Allantoic Fluid Containing ^{59}Fe-Uf Incubated to 96 h *In Vitro*

^{59}Fe-Uf was incubated up to 96 h in pooled Day 60 allantoic fluid previously centrifuged and filter sterilized at 37°C. Aliquots were removed, frozen at -20°C until analyzed, thawed, made 0.4 M in NaCl by adding solid NaCl, the radioactivity determined and loaded onto Sephadex G-100 columns (1.5 x 60 cm). Columns were equilibrated and eluted in sodium barbital-sodium chloride buffer (0.2 M, 0.4 M, pH 7.6). Fractions of 1.1 ml were collected and the radioactivity determined.


Control
U_f
24h
48h
A
B
C
D
E
20
40
60
80
100
120
140
Fraction Number
1
2
^{59}Fe (cpm x 10^{-3})

their isoelectric points and molecular weights. Two methods were employed: 1) standard 2D-PAGE essentially as described by O'Farrell (1975) as modified by Horst et al. (1980) for proteins with isoelectric points between pH 4 and 7.5 and 2) NEPHGE as described by O'Farrell et al. (1977) for resolution of basic proteins. Figure 3-8 shows the Coomassie stained two-dimensional gels of allantoic fluid from different days of gestation (Days 30, 45, 60, 75, 90 and 105). Major proteins identified on these gels by co-migration and by addition of a trace of the iodinated standard to allantoic fluids are the porcine serum proteins, transferrin (Tf), albumin (Alb.), and α-fetoprotein (AFP). Other proteins are present in smaller amounts. Some of these unidentified proteins apparently match with serum Coomassie-stained spots and may include ceruloplasmin, α_2-macroglobulin, α_1 acid glycoprotein, α_1 anti-trypsin, and fetuin. Two acidic uterine proteins found in uterine flushings from progesterone stimulated animals previously identified by Basha et al. (1980) are seen in the allantoic fluids (arrows). Further examination of these gels revealed that the complement of acidic polypeptides does not show any major qualitative changes during pregnancy (Day 30 through Day 105). However, there are clear variations in the relative amounts of transferrin, albumin, and α-fetoprotein and the various unidentified proteins present in allantoic fluid from animals from the same stage of pregnancy (gels not shown).

The proteins present in Day 60 amniotic fluid are very similar to those present in Day 60 fetal serum (Figure 3-9). The three same major proteins (transferrin, albumin and α-fetoprotein) are clearly evident. Major differences between maternal and fetal serum are the absence of the heavy and light chains of immunoglobulins, the low levels

Figure 3-8. Two Dimensional Electrophoresis of Allantoic Fluids from Pregnant Gilts at Various Stages of Gestation

Two dimensional polyacrylamide gel electrophoresis, using isoelectric focusing (pH gradient 8 to 4 from left to right) in the first dimension, of allantoic fluid collected from pregnant gilts at various stages of gestation. The polypeptides were stained with Coomassie blue. The allantoic fluids of different days of gestation are as follows: A) Day 30, B) Day 45, C) Day 60, D) Day 75, E) Day 90, and F) Day 105. The letters a, t, and fp on Figure 3-8A represent the positions of porcine albumin, transferrin, and α-fetoprotein, respectively. Arrows identify two acidic uterine proteins usually found in uterine flushings.

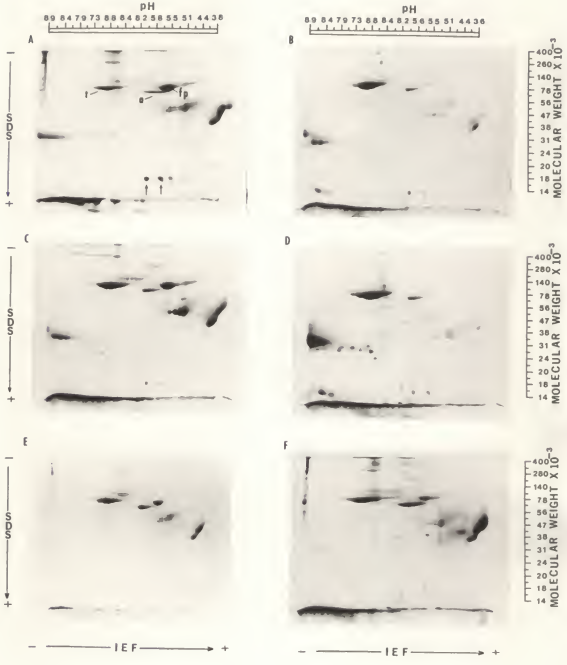
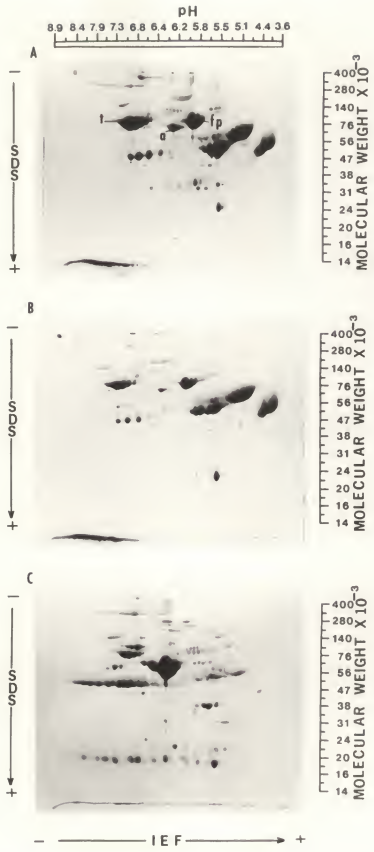

pH
8.9 8.4 7.9 7.3 8.8 8.4 8.2 5.8 5.5 5.1 4.4 3.6
A
B
C
D
E
F
SDS
MOLECULAR WEIGHT X 10⁻³
400
280
140
78
56
47
38
31
24
20
18
14
IEF

Figure 3-9. Two Dimensional Electrophoresis of Amniotic Fluid, Fetal Serum, and Maternal Serum

Two dimensional polyacrylamide gel electrophoresis was performed using isoelectric focusing (pH gradient 8 to 4 from left to right) in the first dimension and standard SDS-PAGE in the second dimension. Samples include A) 300 µg Day 60 amniotic fluid, B) 100 µg Day 60 fetal plasma, and C) 300 µg maternal plasma. Polypeptides were stained by Coomassie blue. The letters a, t, and fp on Figure 3-9A represent the positions of porcine albumin, transferrin, and α-fetoprotein, respectively.


pH
8.9 8.4 7.9 7.3 6.8 6.4 6.2 5.8 5.5 5.1 4.4 3.6
A
B
C
SDS
−
+
t
α
fp
MOLECULAR WEIGHT X 10⁻³
400
280
140
76
56
47
38
31
24
20
16
14
IEF

of albumin in fetal serum and the absence of fetuin and -fetoprotein in maternal serum.

The basic proteins of these same samples were analyzed using NEPHGE. These gels (Figures 3-10 and 3-11) show distinct differences between the polypeptide composition of allantoic fluid relative to amniotic fluid and fetal serum and, in addition, show qualitative changes in allantoic fluid throughout the gestation. Specifically, the basic group of polypeptides including uteroferrin, which are characteristic of uterine flushings and are believed to be secretory products of the maternal uterus (Basha et al., 1979; Basha et al., 1980; Roberts and Bazer, 1980b) are present in allantoic fluid and some are found as major components. These include, in addition to uteroferrin, lysozyme which runs close to the dye front and several other polypeptides located just above uteroferrin (higher molecular weight) which have not yet been characterized, plus one that migrates to a more acidic position than uteroferrin and has a somewhat lower molecular weight. Uteroferrin increases from Day 30 to a maximum at Days 60 and 75 and then decreases at the latter stages of pregnancy.

Thus, the two-dimensional electrophoretic analysis reveals that fetal serum proteins are found in both allantoic and amniotic fluid indicating that the three fluid compartments are in equilibrium. Further, transferrin is found in allantoic fluid in major amounts and in concentrations that are apparently comparable to uteroferrin. In addition, the allantoic fluid at all days examined (Days 30-105) contains proteins identical to those secreted by the maternal uterus and to those released by cultures of endometrial explants under the influence of progesterone.

Figure 3-10 Nonequilibrium pH Gradient Electrophoresis (NEPHGE) of Allantoic Fluid from Pregnant Gilts at Various Stages of Gestation

Two dimensional polyacrylamide gel electrophoresis was performed using NEPHGE in the first dimension and standard SDS-PAGE in the second dimension. Allantoic fluid samples from pregnant gilts at the various stages of gestation are as follows: A) Day 30, B) Day 45, C) Day 60, D) Day 75, E) Day 90, and F) Day 105. Polypeptides were stained with Coomassie blue. The letters U and L in Figure 3-10C represent the positions of uteroferrin and lysozyme, respectively.

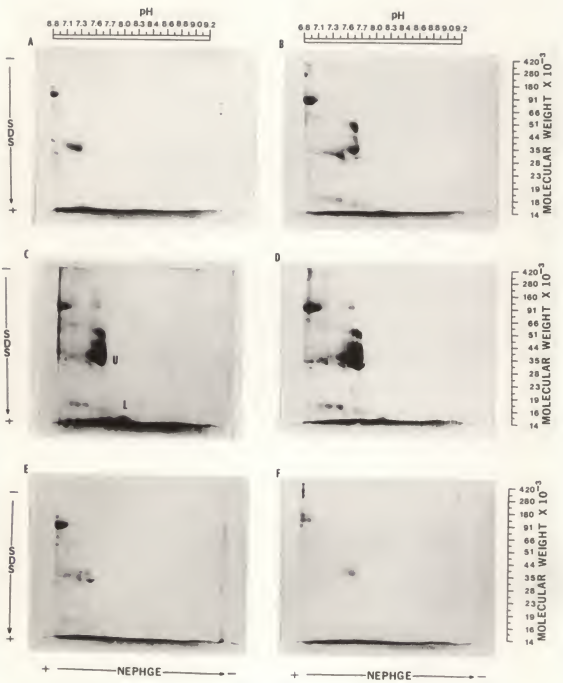
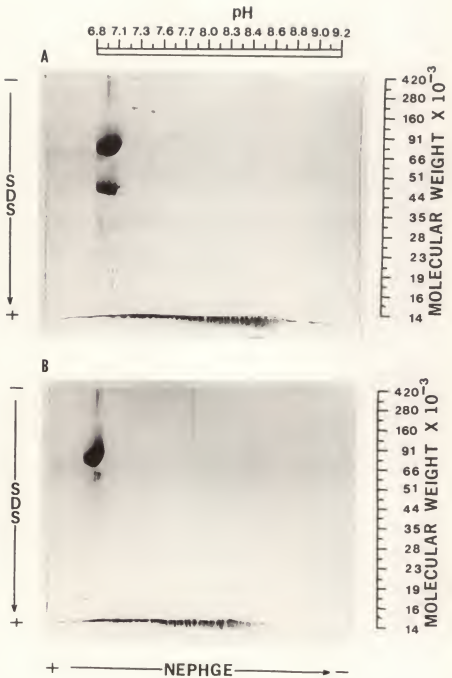

pH
6.8 7.1 7.3 7.6 7.7 8.0 8.3 8.4 8.6 8.8 9.0 9.2
A
B
C
D
E
F
SDS
U
L
MOLECULAR WEIGHT X 10^-3
420 280 160 91 66 51 44 35 28 22 19 16 14
+ NEPHGE −

Figure 3-11 Nonequilibrium pH Gradient Electrophoresis (NEPHGE) of Polypeptides Found in Amniotic Fluid and Fetal Serum at Day 60 of Gestation

Two dimensional polyacrylamide gel electrophoresis was performed using NEPHGE in the first dimension and standard SDS-PAGE in the second dimension. All polypeptides were stained with Coomassie blue. Samples analyzed were A) Day 60 amniotic fluid (Gilt 357, L1) and B) Day 60 fetal plasma (R1).


pH
6.8 7.1 7.3 7.6 7.7 8.0 8.3 8.4 8.6 8.8 9.0 9.2
A
B
SDS
−
+
420
280
160
91
66
51
44
35
28
23
19
16
14
MOLECULAR WEIGHT X 10⁻³
+
NEPHGE
−

Discussion

The development of the conceptus, fetal fluids, and the number of live fetuses through the gestation conforms to previously established patterns as described by Knight et al. (1977) for normal intact swine and requires no further comment here.

To determine the function of uteroferrin during fetal development, it was necessary to analyze its properties in the allantoic fluid. A series of experiments was designed to study the loss of uteroferrin using a number of criteria. These experiments measured the changes in acid phosphatase activity, immunoprecipitability, destruction of protein and loss of ^{59}Fe from uteroferrin.

It was shown earlier by Schlosnagle et al. (1976) that the presence of Fe is not only correlated with the purple color of uteroferrin, but with its acid phosphatase activity. Thus, it is not surprising that the loss of Fe appears to be accompanied by the loss of enzymatic activity. Indeed, since Fe loss seems to follow apparent first order kinetics, it is possible that this event precedes protein destruction. Consistent with this hypothesis are the observations that when allantoic fluids (Days 45-105) are incubated in vitro a) there is a negligible loss of immunological activity of uteroferrin over the first 24 h of the incubations and b) uteroferrin polypeptide destruction is minimal at 24 h. By both criteria there was a more rapid increase in the apparent rate of destruction over the subsequent two days of the incubation.

The results of these observations, namely decreasing acid phosphatase activity, loss of immunoprecipitability, increasing protein destruction, and Fe loss, are consistent with a model in which the Fe is first

lost from the protein followed by rapid proteolysis of the apoprotein. This if further supported by the marked instability of the apoprotein in allantoic fluid.

Using a double-antibody radioimmunoassay for uteroferrin, the concentration (mg/sac) was shown to increase from Day 30 and reach a maximum at Days 60 and 75 and decrease towards parturition. These results correlate very well with previous studies (Bazer et al., 1975; Basha et al., 1979; Roberts, 1980 , unpublished) that show acid phosphatase in allantoic fluid and uteroferrin synthesized by explant cultures of pregnant endometrium to increase to a maximum at Days 60 and 75 and then decrease to parturition. The results from explant cultures (Basha et al., 1979) further indicated that the midpregnant uterus is capable of synthesizing at least 1 gm uteroferrin daily. Thus, the loss of about 40% of uteroferrin Fe in 24 h (from the in vitro incubation of pooled Day 60 allantoic fluid) would indicate a large turnover of uteroferrin and Fe loss within the allantois.

It was somewhat surprising to detect uteroferrin in fetal blood since Chen et al. (1973) using a double immunodiffusion analysis reported none present. However, the RIA most probably detects levels below the sensitivity of the immunodiffusion assay. While Day 60 allantoic fluid contains about 330 µg Uf/ml, fetal cord blood contains only about 2.8 µg/ml, a 100-fold difference, suggesting either a high turnover in blood or low transport from allantoic fluid to fetal blood.

Is allantoic fluid in equilibrium with fetal blood? Both compartments do have a similar protein composition as demonstrated by 2D-PAGE but the allantoic fluid contains much higher levels of uterine secretory products. These products may be selectively excluded from the fetal

blood stream or protected in allantoic fluid from destruction in the liver or other fetal organs.

These studies supported the concept of Fe loss from uteroferrin in allantoic fluid but did not indicate the Fe acceptor. The acceptor could be a low molecular weight chelator since the gel filtration studies indicated a peak of ^{59}Fe in the salt volume. However, this peak did not increase with time but remained relatively constant in amount. Peak B, however, showed a time-dependent accumulation of ^{59}Fe. Further, porcine transferrin eluted in a similar position to this component and its presence as a major polypeptide in allantoic fluid was demonstrated by 2D-PAGE. Moreover, serum transferrin, a known Fe acceptor, is rarely saturated with Fe (Zschocke and Bezkorovainy, 1974). Therefore, porcine transferrin appears to be the potential candidate for the iron acceptor in the conceptus.

Further studies were needed to elucidate the identity of the acceptor and the mechanism of Fe release.

CHAPTER 4
IN VIVO STUDIES OF UTEROFERRIN TURNOVER AND FE DISPOSITION

Introduction

During pregnancy in the pig, uteroferrin has been shown to accumulate in the allantoic fluid through Days 60-75 of gestation and decline thereafter (Bazer et al., 1975; Chen et al., 1975). It is synthesized in the surface and glandular epithelium of the uterus under the control of progesterone and is taken up by the placenta at special regions called areolae (Chen et al., 1973; Chen et al., 1975). Roberts and Bazer (1980) proposed that the primary function of uteroferrin is to transport iron from the mother to the conceptus during pregnancy. In CHAPTER 3 it was shown that uteroferrin loses its acid phosphatase activity and is proteolytically destroyed over time when incubated in allantoic fluid. Further, iron is lost at a rate, that was approximately first order, to a protein tentatively identified as transferrin.

A number of questions were raised by the previous studies, some of which are addressed here. Among these are: does uteroferrin lose its iron in vivo in a manner similar to that noted in vitro and, if it does, what is the acceptor? Following loss of iron from uteroferrin, is the apoprotein degraded? Finally, what is the fate of the iron and its acceptor and is the iron distributed to fetal tissues? Ultimately, our aim was to determine whether the rate of uteroferrin turnover and iron release was consistent with the proposed role of the protein in iron transport in pregnancy.

Materials and Methods

Materials

Carrier-free $Na^{125}I$ was obtained from Union Carbide (17 mC/μg I) or from the Amersham Corporation (13-17 mC/μg I). $^{59}FeCl_3$ (7-9 mCi/mg Fe) was purchased from the Amersham Corporation. All inorganic chemicals were reagent grade or better.

Animals

Sexually mature crossbred gilts were checked daily for estrus in the presence of intact boars. The animals were bred and day of onset of estrus was designated Day 0. Animals used were 58-67 days pregnant. Feed and water were withheld 24 h prior to surgery. The animals were moved to an isolated and controlled location previously approved for animal experiments with radioactive isotopes. At surgery, anesthesia was induced with a 5% solution of sodium thiomylal given intravenously and maintained with Metofane. The reproductive tract was exposed by mid-ventral laparotomy and the uterus arranged outside the abdomen. Labelled materials were injected into the allantois, identified by the color of the fluid, via a 21 gauge needle and syringe through the uterine wall, the chorioallantoic membranes (the placenta) and into the allantoic sac (Figure 1). At 1, 2, and 6 h after injection, the uterus was reexposed and, using a 21 gauge needle and a 10 ml syringe, allantoic fluid was withdrawn. Amniotic fluid was collected in a similar manner. All samples were frozen immediately and stored at -20°C until analyzed. At 24 h after injection, the experiments were terminated and the animal was anesthetized and hysterectomized with the removal of the ovaries, oviduct, uterus, and part of the cervix. The excised reproductive tract was chilled on ice. The uterus was then dissected open to expose

each conceptus, i.e. fetus, placental membranes, and fetal fluids. Final fluid samples were collected as above and the fetus dissected open and the tissue samples (liver, spleen, kidneys, bone and a portion of lung) were removed. The tissues were rinsed free of blood, blotted dry, weighed and frozen at -20°C until analyzed.

Uteroferrin Purification

Uteroferrin was purified from fresh allantoic fluid or uterine flushings as described previously in CHAPTER 3 according to Roberts and Bazer (1980b).

Porcine Transferrin Purification

Porcine transferrin was purified by a procedure based on that of Hristic and Mousesijan (1974). Adult female pig blood was collected, allowed to clot overnight at 4°C and centrifuged at 2250 x g, 4°C, for 30 minutes. The supernatant fraction was saved and adjusted to a pH of 8.5 with 0.1 N NaOH. Any precipitate that formed was removed by centrifugation (2250 x g, 4°C, 30 min). Norit A charcoal (1.2 g/100 ml serum) was added slowly and stirred for several hours at room temperature to remove small molecular weight components. The charcoal was collected by filtration through Whatman No. 1 filter paper. An equal volume of saturated ammonium sulfate was slowly added to the stirred filtrate. The preparation was stirred gently for 2-4 h and the precipitate which formed removed by centrifugation at 2250 x g and discarded. The supernatant fraction was dialyzed overnight against several changes of Tris-HCl buffer (0.01 M, pH 7.8, 4°C). The dialyzate was applied to a column of DEAE-cellulose (4 x 20 cm) previously equilibrated in the Tris-HCl buffer. Transferrin was eluted

using a linear salt gradient (0 - 0.2 M NaCl in the loading buffer) between about 0.05 M and 0.09 M NaCl.

Absorbance

Column effluents were monitored by absorbance at 280 nm to detect peptide material, at 545 nm to follow uteroferrin elution, at 463 nm to detect transferrin, and at 410 nm to detect Soret Bands. The latter serves to measure the degree of blood contamination in the preparations.

Apo-Uteroferrin Preparation

Prepared as described in CHAPTER 3.

Preparation of ^{59}Fe-Uteroferrin

Prepared as described in CHAPTER 3.

Preparation of ^{59}Fe-Transferrin

Porcine apo-transferrin was prepared according to the method of Aisen et al. (1966) by successive dialysis of transferrin against several changes of sodium citrate-sodium acetate buffer (0.1 M, 0.1 M, pH 4.5), iron-free distilled water, and finally against sodium barbital (0.07 M, pH 9.4). All reagents were prepared in Chelex-treated water.

^{59}Fe-transferrin was prepared from apo-transferrin by the method of Halton et al. (1977) in which 0.05 mC ^{59}Fe in sodium citrate (0.15 M) was added to the apo-transferrin (2 mg) in sodium barbital (0.2 M, pH 8.4). The preparation was allowed to stand overnight at room temperature. To remove the free-^{59}Fe from ^{59}Fe-labeled transferrin, the mixture was applied to a Sephadex G-50 column (1.5 x 30 cm) equilibrated and eluted with sodium barbital-sodium chloride buffer (0.02 M, 0.4 M, pH 8.4). One milliliter fractions were collected and the radioactivity monitored.

Iodination of Uteroferrin and Transferrin

The iodination was performed using the Iodo-Gen technique described in CHAPTER 3. Uteroferrin (0.09 - 3 mg) in MES buffer (1 ml, 0.025 M, pH 6.8) and porcine transferrin (0.1 - 1.2 mg) in MES buffer (1 ml, 0.025 M, pH 6.8) or sodium barbital-sodium chloride (0.02 M, 0.4 M, pH 7.8) were added to the Iodo-Gen tube. Carrier-free $Na^{125}I$ (0.5 - 3 mC) was added and the tube shaken for a few seconds every minute for 8 - 15 minutes. Separation of labelled protein from free-^{125}I was also as described in CHAPTER 3.

Gel Filtration

Allantoic fluids and fetal serum samples containing radioactive isotopes were thawed, centrifuged (2250 x g, 4°C, 10 -15 min), the supernatants saved and counted, and solid NaCl added to make the sample 0.4 M. These solutions were then loaded onto columns of Sephadex G-100 (1.5 x 75 - 86 cm) or Sephacryl S-200 (1.5 x 96 cm) previously equilibrated with barbital-saline buffer (0.02 M, 0.4 M, pH 7.8). Elution was performed with the same buffer and the fractions (1.1 ml) collected and monitored by following the radioactivity. Calibration of the columns was performed using the following standards: Blue Dextran, ^{125}I-transferrin, ^{125}I-uteroferrin, ^{59}Fe-transferrin and ^{59}Fe-uteroferrin.

Extraction and Determination of Fetal Hemoglobins

Fetal cord bloods were collected by syringe, placed in heparinized tubes and chilled on ice. Plasma was separated from cells by centrifugation (2250 x g, 4°C, 10 - 15 min). Plasma was removed and frozen for later use and cell lysis carried out by the procedure of Gabbay et al. (1977). Briefly, the cells were washed two times with ice-cold saline

(0.85%, w/v), centrifuged (325 x g, 4°C, 5 min), rewashed and centrifuged the last time at 900 x g. The washed cells were swollen by adding 1.5 volumes of ice-cold water and hemolysis completed by gently rotating the tubes in an ice-bath for 5-10 minutes. The lysate was then extracted with 0.5 volume (original packed cell volume) of toluene and the tube shaken vigorously for 5 minutes. The mixture was centrifuged (2250 x g, 4°C, 30 min) and the aqueous layer removed. This suspension was centrifuged (17,000 x g, 5°C, 45 min) and the supernatant fraction containing the hemoglobin collected. A portion of this was brought to 1x Buffer #6 concentration (4.59 g $NaH_2PO_4 \cdot H_2O$, 1.18 g Na_2HPO_4, 0.65 g KCN in one liter distilled water, pH 6.72) by addition of concentrated (5x) buffer and applied to a Bio-Rex 70 ion-exchange column (a 12 ml syringe packed to the 10 ml level with resin). The column was previously equilibrated with Buffer #6 until the pH was 6.72. Fractions (1.1 ml) were collected using the loading buffer as eluant, but after collecting 70 fractions, the hemoglobin was eluted with sodium phosphate buffer (0.15 M, pH 6.42). Fractions were monitored for radioactive content and absorbance at 415 nm.

Trichloroacetic Acid (TCA) Precipitation of Proteins

Ice-cold 50% (w/v) trichloroacetic acid (1 ml) was added to samples of soluble protein (10 µl - 1 ml) on ice containing a carrier protein (0.1 ml, 0.1% w/v, BSA). After 1 h on ice the samples were centrifuged (2250 x g, 4°C, 15 min), the supernatant fraction saved for determination of its radioactivity and the precipitates washed with ice-cold 50% (w/v) trichloroacetic acid (2 ml). The second supernatant fraction was discarded and the pellet and first supernatant counted to determine the quantities of radioactive protein precipitated.

Anti-Porcine Transferrin Antibody Production

Porcine transferrin (1 mg) in physiological saline (0.5 ml) and Freund's complete adjuvant (0.5 ml) were emulsified and injected subdermally in a White New Zealand male rabbit once every seven days for three consecutive weeks. The rabbit was bled about 14 days after the last injection. The blood was allowed to clot overnight at 4°C, centrifuged (2250 x g, 4°C, 30 min) and the serum saved and titered. Further bleeding and booster injections were carried out periodically.

Immunoprecipitation of Transferrin

The sample was diluted to 1 ml in Tris-HCl buffer (0.08 M, pH 7.5) and 0.1 ml of rabbit serum directed against porcine transferrin added. After 48 h at 4°C, 0.1 ml rabbit γ-globulin (400 ng/ml) followed by 0.1 ml of goat anti-rabbit γ-globulin (diluted 1:4 with 0.02 M sodium barbital, 0.15 M sodium chloride, pH 7.8) were added and allowed to incubate for 24 h at 4°C. The samples were centrifuged (2250 x g, 4°C, 15 min), and the supernatant solution decanted. The pellet was washed with cold barbital-saline buffer (0.02 M, 0.4 M, pH 7.8), recentrifuged as above, and the pellet collected. The percentage of transferrin immunoprecipitated by this procedure was calculated from recoveries of radioactivity in the pellet and in the first supernatant.

Immunoprecipitation of Fetal Serum Transferrin from Column Eluate

After fractionation of fetal serum for content of radioactive (^{125}I) transferrin, each fraction from the gel filtration column was divided into two 0.5 ml aliquots. Porcine transferrin (25 µl; 29 µg) was added to one of these fractions and 0.02 M barbital- 0.4 M saline buffer (25 µl, pH 7.8) to the other. All samples then received 25 µl of whole rabbit serum directed against whole porcine serum and were

incubated for 48 h at 4°C. Following this, whole goat anti-rabbit γ-globulin (25 µl) was added and incubated for a further 24 h at 4°C. All samples were centrifuged (2250 x g, 4°C, 20 min) and the supernatant fraction decanted and saved. The pellet was washed with cold barbital-saline buffer (0.02 M, 0.4 M, pH 7.8), recentrifuged as above, and collected for estimation of radioactive content with the first supernatant.

Results

Metabolism of ^{59}Fe-Uteroferrin In Vivo in the Allantoic Sac of Pregnant Gilts

In this study we wished to determine whether ^{59}Fe from uteroferrin was transferred to the putative iron acceptor transferrin in allantoic fluid *in vivo* in a manner analogous to that seen during *in vitro* experiments. Further, we wished to study the rate of transfer, to examine the fate of the iron and to determine if it was ultimately distributed to fetal tissues. ^{59}Fe-uteroferrin was prepared and injected into selected allantoic sacs of four surgically prepared pregnant gilts (149G, 357, 69B and # unknown) between 58 and 67 days of pregnancy. At various times during the next 24 h, samples of allantoic fluid were withdrawn under sterile conditions and the loss of ^{59}Fe from uteroferrin monitored by gel filtration of the samples on columns of either Sephadex G-100 or Sephacryl S-200 (Figure 4-1). As with the *in vitro* experiments described in CHAPTER 3, the ^{59}Fe was lost from uteroferrin in a time-dependent manner and appeared primarily in increasing amounts in a protein of higher molecular weight. When the percent ^{59}Fe loss from uteroferrin was determined in relation to time after injection, it was apparent that the kinetics are approximately first order (Figure 4-2). The half-life of ^{59}Fe-uteroferrin is slightly less than

Figure 4-1. Sephadex G-100 Chromatography of Allantoic Fluid from a Pregnant Gilt After Injection of ^{59}Fe-Uteroferrin into the Allantoic Sac

^{59}Fe-uteroferrin was injected into the allantoic sac of a pregnant gilt (69B, Day 60 of gestation, sac L1) and allantoic fluid was withdrawn 2, 6, and 24 h later. After centrifugation, counting in a γ-counter, samples (1.5 ml) were made 0.4 M with respect to NaCl by adding solid NaCl. The allantoic fluids were loaded onto a Sephadex G-100 column (1.5 x 75 cm) equilibrated and eluted with sodium barbital-sodium chloride buffer (0.02 M, 0.4 M, pH 7.8). Fractions of 1.1 ml were collected and plotted against the radioactivity (cpm). Blue dextran, ^{125}I-labelled porcine transferrin and uteroferrin were used as markers.

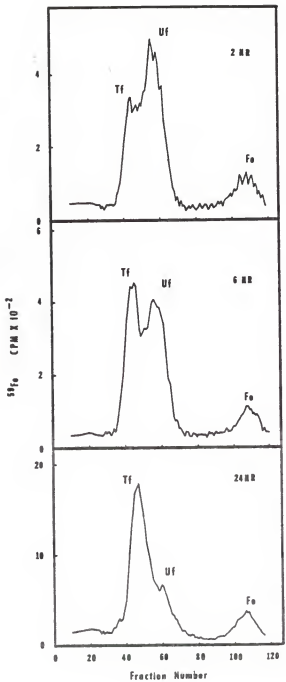
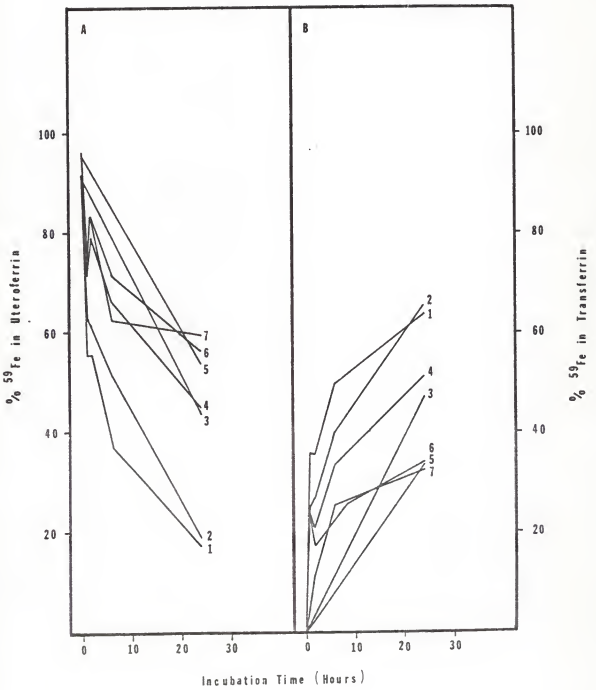

Uf
2 HR
Tf
Fo
Tf
Uf
6 HR
Fo
Tf
24HR
Uf
Fo
^{59}Fe CPM X 10^{-2}
Fraction Number

Figure 4-2. A. Loss of Iron from ^{59}Fe-uteroferrin with Time After its Injection into Allantoic Fluid of Day 60 Pregnant Gilts

B. Accumulation of ^{59}Fe in Transferrin with Increasing Time After Injection of ^{59}Fe-uteroferrin into Allantoic Fluid of Day 60 Pregnant Gilts

Four pregnant gilts (between 58 and 67 days of gestation) were injected with ^{59}Fe-uteroferrin into selected allantoic sacs and samples of allantoic fluid withdrawn at various times through 24 h and frozen immediately at -20°C. Before use, the fluids were thawed, centrifuged (2250 x g, 4°C, 10-15 min), made 0.4 M in NaCl and fractions loaded on either Sephadex G-100 or Sephacryl S-200. Fractions of 1.1 ml were collected and the radioactivity determined. The percent ^{59}Fe in uteroferrin and transferrin was calculated and plotted against the time of incubation. Pregnant gilts, days of gestation, allantoic sac injected, and number of samplings taken for each experiment are as follows: 1) 69B, Day 60, R1,(5); 2) 69B, Day 60, L1,(5); 3) Unknown #, Day 67, R2,(2); 4) 149G, Day 60, L1,(5); 5) 357, Day 58, L4,(2); 6) 149G, Day 60, R1,(5); and 7) 357, Day 58, L1, (5).


A
B
100
80
60
40
20
0
10
20
30
% 59Fe in Uteroferrin
% 59Fe in Transferrin
Incubation Time (Hours)
7
6
5
4
3
2
1

24 h with a mean ± SEM of 42.3 ± 6.6% ^{59}Fe-uteroferrin remaining at 24 hours. Note, however, the large variation between the allantoic sacs from different animals. The iron-acceptor, tentatively identified by its chromatographic properties as transferrin, contained a mean ± SEM of 46.8 ± 5.3% of the total ^{59}Fe recovered at 24 hours. This protein co-elutes on Sephadex G-100 or Sephacryl S-200 with ^{125}I-transferrin. In addition no smaller molecular weight peaks were found with the exception of one at the salt volume (which is assumed to be free-^{59}Fe or ^{59}Fe bound to a low molecular weight chelator). The amount of ^{59}Fe in the low molecular weight fraction remained fairly constant throughout the incubation.

The ^{59}Fe-labelled, high molecular weight peaks (putative transferrin) from several gel filtration columns were pooled, dialyzed, lypholyzed and redissolved in buffer. This sample was treated first with rabbit anti-porcine transferrin antibody and then with goat anti-rabbit γ-globulin to establish that this was definitely porcine transferrin and to eliminate the possibility that this was dimeric uteroferrin. Although the resulting precipitate contained only 30-44% of the ^{59}Fe-label, optimum conditions were not established for immunoprecipitation. Moreover, the control, using non-immune serum contained only 2.7 - 8.5% of the ^{59}Fe. These results strongly suggest that transferrin is the acceptor of Fe from maternal uteroferrin. In addition, Ouchterlony plates (immunodiffusion analysis) have previously been used to show that there was no cross reactivity between porcine transferrin, lactoferrin and uteroferrin with rabbit anti-porcine transferrin antibody (CHAPTER 3).

The distribution of ^{59}Fe in the fetal tissues and fluids is shown in Table 4-I. Little radioactivity was found in amniotic fluids even

Table 4-I

Radioactivity in Fetal Fluids and Tissues 24 Hours After the Injection of ^{59}Fe-uteroferrin into the Allantoic Sacs of Days 58-67 Pregnant Gilts

Animal Number	Allantoic Sac Injected	Whole Blood	Allantoic Fluid	Amniotic Fluid	Total Packed Cells	Total Plasma
		cpm/ml			cpm/ml	cpm/total volume
69B	L1	1660	23160	130	235	122
	R1	1150	3770	130	1284	155
unknown	R2	990	1355	0	1760	114
357	L1	156	2110	0	38	6
	R1	140	3080	ND*	104	6
149G	R1	1010	11290	0	ND	ND
	L1	30	16540	0	ND	ND

Animal Number	Allantoic Sac Injected	Spleen	Lung	Bone	L. Kidney	R. Kidney	Liver
		cpm/gm wet weight					
69B	L1	576.9	72.1	542.3	{949}		2985.2
	R1	3681.1	69.1	425.8	{120}		2588.5
unknown	R2	1692.8	86.5	88.2	1420	157.6	1042.3
357	L1	668	18.6	56	44	44	1604.0
	R1	656	27	0	89	85	1386
149G	R1	3682.5	166.3	89.1	308.1	470.4	2045.4
	L1	3515.2	177.3	63.2	236.1	273.2	2044.8

ND -- Not Determined

^{59}Fe-uteroferrin was injected into selected allantoic sacs of living fetuses of pregnant gilts (69B, 149G, 357, and unknown #) between 58 and 67 days of pregnancy. At 24 h after the injection, the experiment was terminated and samples collected. Fetal cord blood samples were collected and placed in heparinized tubes on ice. Later, the plasma was separated from the cells by centrifugation (2250 x g, 4°C, 10-15 min), the cells washed with cold saline (0.85%) and the radioactivity in both the cells and plasma determined. Amniotic and allantoic fluids were collected and frozen at -20°C, thawed later, centrifuged and the supernatants counted. Tissues were removed from the fetuses, rinsed in distilled water, blotted dry, weighed and frozen at -20°C. The allantoic sac letters L and R refer to the left and right uterine horns, respectively, while the number refers to the position of the fetus in the uterine horn, one being closest to the tubo-uterine junction.

after 24 hours. However, fetal liver, spleen, and sometimes kidney appear to sequester large amounts of ^{59}Fe. Fetal cord blood also contained considerable amounts of the isotope. When the fetal cord blood was separated into its components, total packed cells and total plasma, the majority of the radioactivity (66-95%) was found in the former. When the erythrocytes from fetal cord blood (gilt 69B, Day 60, fetus R1) were extracted and analyzed for hemoglobin by the method of Gabbay et al. (1977), all the ^{59}Fe in the red cells was recovered in the major hemoglobin fraction (Figure 4-3). This indicated that the iron from uteroferrin had, by an unknown route, but possibly involving transferrin, entered the fetal circulation and had become incorporated into fetal hemoglobin.

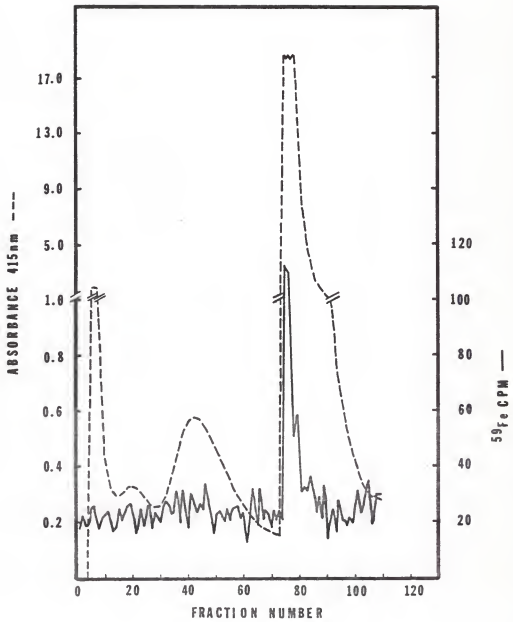
Metabolism of ^{59}Fe-Transferrin In Vivo in the Allantoic Sac of a Pregnant Gilt

In the previous section it was demonstrated that after ^{59}Fe-uteroferrin was injected into the allantoic fluid of a living fetus, iron a) appeared in a molecule resembling transferrin in the allantoic sac and b) was transferred to fetal tissues. A likely explanation was that transferrin is the intermediary in this movement to the fetus. This hypothesis was tested by injecting ^{59}Fe-transferrin into allantoic fluid and the deposition of its ^{59}Fe followed.

The ^{59}Fe-transferrin was injected into two allantoic sacs of a surgically prepared pregnant pig (gilt 333, 60 days of gestation). At various time periods up to 24 h after this treatment samples of allantoic fluid were withdrawn and the distribution of ^{59}Fe monitored by gel filtration on Sephadex G-100. Analysis at all time periods indicated only two radioactive peaks, one corresponding to the elution position of transferrin and the second eluting in the salt volume. The latter

Figure 4-3. Elution Profile of Total Hemoglobins From Bio-Rex 70 After Extraction From Day 60 Fetal Erythrocytes

Fetal cord blood (gilt 69B, Day 60, fetus R1) was collected in heparinized tubes and placed on ice 24 h after the injection of ^{59}Fe-uteroferrin into the allantoic sac of a pregnant gilt. Blood was centrifuged, the erythrocytes saved and washed three times with cold saline (0.85%) and extracted according to the method of Gabbey et al. (1977). The supernatant (containing the hemoglobin) was brought to 1x Buffer #6 concentration by the addition of concentrated (5x) buffer and applied to a Bio-Rex 70 ion-exchange column. Fractions (1-70) of 1.1 ml were eluted with Buffer #6, pH 6.72, the buffer changed to sodium phosphate (0.15 M, pH 6.42), and the elution continued. The absorbance of the hemoglobin fractions at 415 nm (---) and radioactivity (__) was determined.


17.0
13.0
9.0
5.0
1.0
0.8
0.6
0.4
0.2
ABSORBANCE 415nm ---
120
100
80
60
40
20
59Fe CPM —
0
20
40
60
80
100
120
FRACTION NUMBER

may be free Fe that was not removed from the ^{59}Fe-transferrin when purified by gel filtration. No ^{59}Fe was associated with a uteroferrin fraction, indicating that Fe exchange between uteroferrin and transferrin is not bidirectional. Thus, it appears that uteroferrin transfer of iron to transferrin is a one-way transfer and not reversible under the in vivo conditions.

The data in Table 4-II show the distribution of ^{59}Fe recovered in fetal tissues and fluids. They indicate that ^{59}Fe was taken up by the fetal tissues and especially fetal blood (fetus R1). The tissue distribution is very similar to that observed after supplying ^{59}Fe-uteroferrin to allantoic fluid with the liver and spleen sequestering the metal. The activity in fetal blood (fetus R1) compared to the allantoic fluid would indicate a rapid transfer of Fe from the fluid to fetal circulation. The fetal cord blood samples were not of sufficient volume to analyze for hemoglobin ^{59}Fe content as above.

Metabolism of ^{125}I-Uteroferrin In Vivo in the Allantoic Sac of Pregnant Gilts

Since ^{59}Fe from both uteroferrin and transferrin is rapidly transferred from the allantoic fluid to fetal tissues, it was important to determine whether the protein itself entered the fetal blood stream and thus, participated directly in the transfer. To test this question, the polypeptide portion of the protein was labelled with ^{125}I and injected into allantoic fluid.

With ^{125}I-uteroferrin, three pregnant gilts (#446, #333, and #unknown) between 60 and 67 days of gestation were injected as previously described and samples were withdrawn from the allantoic sac over the 24 h of the experiments. As shown in Figure 4-4 for the 24 h sample, no new species of

Table 4-II

Radioactivity in Fetal Tissues and Fluids 24 h After the Injection of ^{59}Fe-transferrin into the Allantoic Sacs of a Day 60 Pregnant Gilt

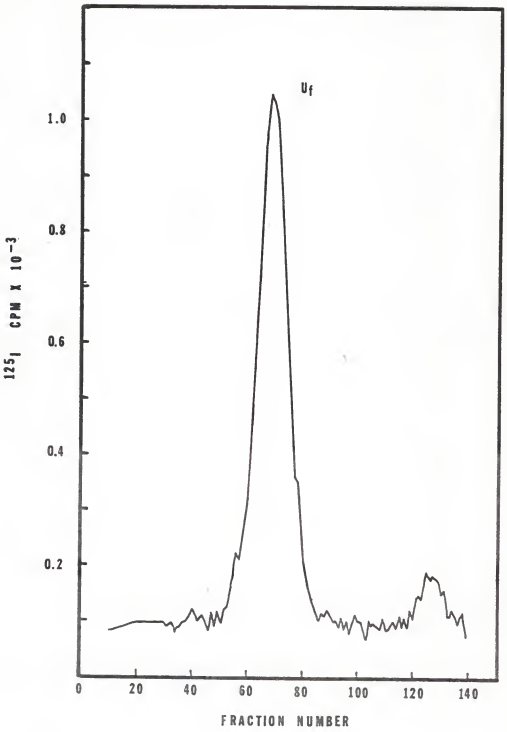
Animal Number	Allantoic Sac Injected	Whole Blood	Allantoic Fluid	Amniotic Fluid
		cpm/ml		
333	L3	760	4490	50
	R1	2593	1560	0

Animal Number	Allantoic Sac Injected	Spleen	Lung	Bone	L. Kidney	R. Kidney	Liver
		cpm/gm wet weight					
333	L3	851.4	71.2	122.1	125.9	132.9	1263.2
	R1	4137.9	160.3	427.0	356.8	416.2	5087.8

^{59}Fe-transferrin was injected into the allantoic sacs of fetuses 1 and 3 in the right (R) and left (L) uterine horns, respectively, of a Day 60 pregnant gilt (#333). The experiment was terminated and samples collected 24 h after the injection. Blood samples were placed in heparinized tubes on ice. Amniotic and allantoic fluids were collected and frozen at -20°C, thawed later, centrifuged and the supernatants counted. Tissues were removed from the fetuses, rinsed in distilled water, blotted dry, weighed and frozen at -20°C until the radioactivity was determined.

Figure 4-4. Sephadex G-100 Chromatography of an Allantoic Fluid Sample From a Day 60 Pregnant Gilt 24 h After the Injection of ^{125}I-uteroferrin into the Allantoic Sac

A pregnant gilt (#333, at 60 days of gestation) was injected with ^{125}I-uteroferrin into the allantoic sac L4 and allantoic fluid withdrawn at various times through 24 hours. After centrifugation and counting in a γ-counter, the 24 h allantoic fluid sample (1.0 ml) was made 0.4 M in NaCl by adding solid NaCl. The fluid was loaded on a Sephadex G-100 column (1.5 x 85 cm) equilibrated and eluted with sodium barbital-sodium chloride buffer (0.02 M, 0.4 M, pH 7.8). Fractions (1.1 ml) were collected and the radioactivity determined. Blue dextran, ^{125}I-labelled porcine transferrin and uteroferrin were used as markers.


U_f
1.0
0.8
0.6
0.4
0.2
^{125}I CPM X 10^{-3}
0
20
40
60
80
100
120
140
FRACTION NUMBER

iodinated polypeptides could be detected by gel filtration on Sephadex G-100 over that time period. The major peak that was present eluted in a position that corresponded to ^{125}I-uteroferrin (>90% of the radioactivity) and the second smaller peak (<10% of the radioactivity) appeared in the salt volume. This salt volume peak was presumably free ^{125}I, monoiodotyrosine or small peptides.

Table 4-III lists the distribution of radioactivity in the fetal tissues and fluids. The allantoic fluid samples L1, L3, and L4 gave 88.1%, 79.6%, and 67.5% precipitation of radioactivity, respectively, with trichloroacetic acid. The amniotic fluids also contained significant amounts of radioactivity. Amniotic fluids L1, L3, and L4 gave a precipitation of 28.3%, 19.8%, and 28.4%, respectively, with trichloroacetic acid. Precipitation of ^{125}I-uteroferrin by trichloroacetic acid from buffer indicated 93.8% was precipitable. The fetus, contrary to the Fe-labelled uteroferrin and transferrin experiments, showed no sequestering of the ^{125}I in any particular tissue. Label was evenly distributed throughout the major organs and was probably mainly in the blood. A portion of fetal liver (fetus L4, gilt #333) was crudely homogenized and precipitated by trichloroacetic acid and only 14.4% of the label was precipitable.

Fetal cord blood also contained considerable amounts of radioactivity. In three specimens in which both cells and plasma were available, the plasma contained the majority of the isotope. A trichloroacetic acid precipitate of the plasma samples from fetuses L1 and L3 revealed that 10.2% and 52.8% of the label was precipitated, respectively. This, in addition to the amniotic fluid precipitate results, indicates that some polypeptide, possibly intact uteroferrin,

Table 4-III

Radioactivity in Fetal Tissues and Fluids 24 h After the Injection of ^{125}I-uteroferrin into the Allantoic Sacs of Day 60-67 Pregnant Gilts

Animal Number	Allantoic Sac Injected	Whole Blood	Allantoic Fluid	Amniotic Fluid	Total Packed Cells	Total Plasma
			cpm/ml		cpm/ml	cpm/total volume
333	L4	1015	18820	910	ND	ND
	R3	720	11320	700	ND	ND
446	L1	240	7090	190	18	40
	L3	180	4000	250	0	32
	L4	70	4230	100	45	ND
unknown	R4	1060	5381	1170	112	384

Animal Number	Allantoic Sac Injected	Spleen	Lung	Bone	L. Kidney	R. Kidney	Liver
		cpm/gm wet weight					
333	L4	468.5	410.4	178.4	475.6	480.7	681.7
	R3	160.9	446.0	153.3	538.5	512.9	675.4
446	L1	350.0	ND	0	{101.6}		224.8
	L3	26.3	ND	166.7	{107.0}		165.9
	L4	48.2	ND	0	{ 64.3}		58.2
unknown	R4	706.1	487.8	516.4	646.7	566.0	658.5

N.D. = Not Determined

^{125}I-uteroferrin was injected into the allantoic sacs of living fetuses in both uterine horns of pregnant gilts (#333, 446, and # unknown) between 60 and 67 days of gestation. At 24 h after the injection the experiment was terminated and all samples collected as previously described. Fetal cord blood samples collected by syringe were placed in heparinized tubes on ice. Later, the plasma was separated from the cells by centrifugation (2250 x g, 4°C, 10-15 min), the cells washed with cold saline (0.85%) and the radioactivity in both the plasma and cells determined. Amniotic and allantoic fluids were collected and frozen at -20°C, thawed later, centrifuged and the radioactivity in the supernatants determined. Tissues were removed from the fetuses, rinsed in distilled water, blotted dry, weighed and frozen at -20°C until the radioactivity was determined.

is present in fetal serum and amniotic fluid and has escaped the allantoic sac. However, the low specific activity of the blood compared with the allantoic fluid suggests little transport from allantoic sac to the fetus had occurred. Clearly, the majority of the iodine outside the allantoic sac was not in polypeptide material.

Metabolism of ^{125}I-Transferrin In Vivo in the Allantoic Sac of a Pregnant Gilt

The experimental results presented earlier suggest that porcine transferrin is the acceptor of Fe from uteroferrin and is the mediator of Fe transport from the placenta and/or allantoic fluid to the fetus. Thus, it is necessary to examine whether transferrin moves readily from the allantoic fluid to fetal blood. The experimental design was to inject iodinated porcine transferrin in vivo into the allantois of a 60 day pregnant gilt (69B) and examine its distribution after 24 hours. Samples of allantoic fluid were withdrawn at various times during the 24 h following the injection and were examined by gel filtration on Sephadex G-100. No new iodinated peaks could be detected through the 24 h (Figure 4-5). Only a high molecular weight peak that corresponded to ^{125}I-transferrin was observed. This contained about 97% of the radioactivity. The remainder was recovered in a small peak appearing in the salt volume (<3% of the recovered radioactivity). This latter peak may represent free radioactive iodine since it is also seen with freshly prepared ^{125}I-transferrin.

Table 4-IV lists the distribution of radioactivity associated with the fetal tissues and fluids. The fetal cord blood (fetus L3) contained very high amounts of radioactivity. When the plasma was separated from the cells by centrifugation, the majority of the isotope (96.8%) was recovered in the former. An aliquot of this fetal plasma was subjected

Figure 4-5. Sephadex G-100 Chromatography of an Allantoic Fluid Sample From a Day 60 Pregnant Gilt 24 h After the Injection of ^{125}I-transferrin into the Allantoic Sac

A pregnant gilt (69B, at 60 days of gestation) was injected with ^{125}I-transferrin into the allantoic sac L3 and allantoic fluid withdrawn at various times through 24 hours. After centrifugation and counting in a γ-counter, the 24 h allantoic fluid sample (1 ml) was made 0.4 M in NaCl by adding solid NaCl. The fluid was loaded on a Sephadex G-100 column (1.5 x 85 cm) equilibrated and eluted with sodium barbital-sodium chloride buffer (0.02 M, 0.4 M, pH 7.8). Fractions (1.1 ml) were collected and the radioactivity determined. Blue dextran, ^{125}I-labelled porcine transferrin and uteroferrin were used as markers.

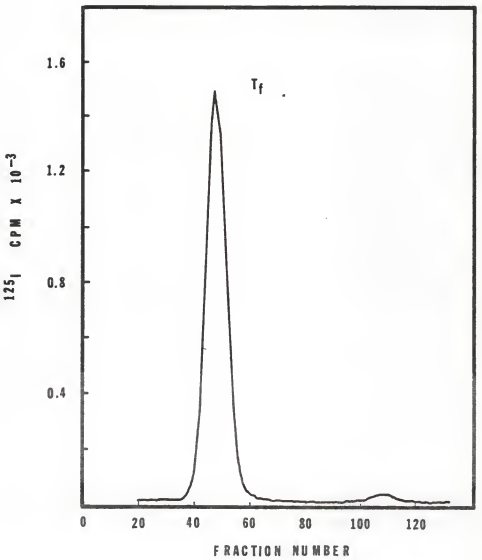

1.6
1.2
0.8
0.4
^{125}I CPM X 10^{-3}
T_f
0
20
40
60
80
100
120
FRACTION NUMBER

Table 4-IV

Radioactivity in Fetal Tissues and Fluids 24 h After the Injection of ^{125}I-transferrin into the Allantoic Sac of a Day 60 Pregnant Gilt

Animal Number	Allantoic Sac Injected	Total Plasma	Total Packed Cells	Whole Blood	Allantoic Fluid	Amniotic Fluid
		cpm/total volume	cpm/ml		cpm/ml	
69B	L3	4551	151	5150	14937	630
	R2	ND	ND	ND	28970	220

Animal Number	Allantoic Sac Injected	Spleen	Lung	Bone	Kidneys	Liver
				cpm/gm wet weight		
69B	L3	576.3	633.7	549.5	513.1	556.7
	R2	111.1	262.2	0	300.8	447.7

ND = Not Determined

^{125}I-labelled porcine transferrin was injected into the allantoic sacs of fetuses R2 and L3 (the right and left uterine horns, respectively) of a pregnant gilt (69B) at 60 days of gestation. At 24 h after the injection, the experiment was terminated and samples collected. Fetal cord blood was collected by syringe and placed in heparinized tubes on ice. Later, the plasma was separated from the cells by centrifugation (2250 x g, 4°C, 10-15 min), the cells washed with cold saline (0.85%), and the radioactivity in both plasma and erythrocytes determined. Amniotic and allantoic fluids were collected and frozen at -20°C, thawed later, centrifuged and the radioactivity in each determined. Tissues were removed from the fetuses, rinsed in distilled water, blotted dry, weighed and frozen at -20°C until the radioactivity could be determined.

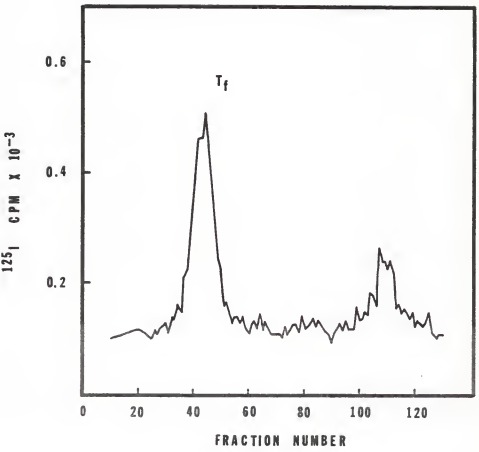
to gel filtration on Sephadex G-100 (Figure 4-6). It gave two peaks of radioactivity. The major peak (76.5% of the recovered radioactivity) eluted in a position that corresponded with transferrin while the smaller one (23.5% of the radioactivity) was found in the salt volume of the column. Samples from the major peak (putative transferrin) were treated first with rabbit anti-porcine whole serum antibody and then with goat anti-rabbit γ-globulin. Identical fractions to which exogenous porcine transferrin (28 μg) was added were treated similarly. Although the immunoprecipitation was not carried out under optimal conditions, 23-33% of the labelled protein in the first group was precipitated. In those samples containing the exogenously added transferrin only 1-3% of the isotope was precipitated. These results suggested that the iodinated porcine transferrin previously injected into the allantoic fluid enters the fetal circulation and that the majority is not degraded but escapes intact and is fully immunoprecipitable.

The distribution of ^{125}I in fetal tissues was similar to that observed with the ^{125}I-uteroferrin experiment. There was a fairly even distribution of the ^{125}I with no apparent sequestration in any particular organ or tissue. Further, radioactivity was again detected in the amniotic fluid although the nature of this material has not been confirmed. Note that the specific activity of the blood was fairly high indicating a ready escape of iodinated transferrin from the allantoic sac.

These results suggest that transferrin is not degraded in allantoic fluid over the time course of the experiment (24 h) but readily enters the fetal blood in intact form.

Figure 4-6. Sephadex G-100 Chromatography of Fetal Plasma 24 h After the Injection of ^{125}I-transferrin into the Allantoic Sac of a Day 60 Pregnant Gilt

^{125}I-labelled porcine transferrin was injected into the allantoic sac (L3) of a Day 60 pregnant gilt (69B). At 24 h after the injection, the experiment was terminated and fetal cord blood collected by syringe and placed in heparinized tubes on ice. The plasma was separated from the cells by centrifugation (2250 x g, 4°C, 10-15 min) and frozen at -20°C. To a 1.1 ml sample, solid NaCl was added to a concentration of 0.4 M, the radioactivity determined and the sample loaded on a Sephadex G-100 column (1.5 x 85 cm). The column had been equilibrated and was eluted with sodium barbital-sodium chloride buffer (0.02 M, 0.4 M, pH 7.8). Fractions of 1.1 ml were collected and the radioactivity determined. Column markers were blue dextran, ^{125}I-transferrin and ^{125}I-uteroferrin.


0.6
0.4
0.2
T_f
^{125}I CPM X 10^{-3}
0
20
40
60
80
100
120
FRACTION NUMBER

Injection of ^{125}I-Porcine Transferrin into the Maternal Blood Stream

In this experiment we wished to know whether maternal transferrin can cross the placental barrier and act in the transport of iron to the conceptus.

^{125}I-transferrin was prepared and injected into the maternal blood stream of a Day 58 pregnant gilt (#326). After 24 h the pregnancy was interrupted and fetal cord blood, allantoic and amniotic fluid samples taken from the first three conceptuses in each uterine horn. As shown in Table 4-V radioactivity had accumulated in these fluids. The amniotic fluid had significantly less radioactivity than did the allantoic fluid. These allantoic and amniotic fluid samples were subjected to both 50% (w/v) trichloroacetic acid precipitation and immunoprecipitation with rabbit anti-porcine transferrin antibody. The results indicated no precipitation by either method above background levels. This suggests that the ^{125}I is no longer present in transferrin nor in peptides of significant size (5-10,000 M_r) which would be expected to be precipitated by trichloroacetic acid. Further, it would suggest that the transferrin in amniotic and allantoic fluid is not of maternal origin and that transferrin does not cross the placental barrier.

Discussion

The *in vivo* studies reported in this chapter appear to give results that are consistent with the *in vitro* experiments. In four midpregnant gilts, Fe was lost from ^{59}Fe-uteroferrin introduced into allantoic fluid in a manner that appeared similar to that observed in pooled Day 60 allantoic fluid experiments (*in vitro*). However, the rate of Fe loss appeared much faster. Indeed the half-life of ^{59}Fe-uteroferrin

Table 4-V

Radioactivity in Fetal Fluids 24 h After the Injection of ^{125}I-transferrin into the Maternal Circulation of a Day 58 Pregnant Gilt

	Allantoic Sac Sampled					
Fetal Fluid	R1	R2	R3	L1	L2	L3
	cpm/ml					
Allantoic	7110	5740	7580	16230	8530	11000
Amniotic	1280	1170	1210	1170	1240	1440
Fetal Cord Blood	2610	ND*	2270	3710	1920	ND

*ND -- Indicates Not Determined

^{125}I-labelled porcine transferrin was injected into the maternal circulation of a pregnant gilt (gilt 326, Day 58 of gestation) through an ear vein. At 24 h after the injection the experiment was terminated and samples of fetal cord blood, allantoic, and amniotic fluid were collected from the first three fetuses in each uterine hron as previously described. Fluids were centrifuged (2250 x g, 4°, 10-15 min) and the radioactivity in each supernatant and cord blood was determined.

in vivo was about one-third (15 h) of that described for the in vitro (50-60 h) work. This may be due to the fact that the whole animal studies constitute an open system, whereas in vitro the system is closed, and there may only be a limited amount of iron binding compounds such as ascorbic acid and transferrin. Further, exchange with the fetal blood supply is, of course, not possible in vitro.

The proteolytic destruction of uteroferrin in allantoic fluid evident during in vitro studies was not evident in the in vivo experiments. For example, gel filtration revealed no iodinated peptides which had been generated during the 24 h experiments. However, it must be recalled that the in vitro experiments were carried out over a period of several days and that little uteroferrin breakdown was noted over the initial 24 hours. The experiments with live animals were terminated after one day because of the likely probability of abortion induced by initial surgeries and the subsequent manipulations of the uterus. Further, it is possible that uteroferrin may be taken up at the allantoic epithelium and transferred to the fetal blood.

The question arises as to whether uteroferrin is in equilibrium with the fetal circulation. The fetal cord blood in the ^{125}I-uteroferrin experiments had a low specific activity compared to that of the allantoic fluid (being between 2.0 - 6.4% except one at 19.7%). This suggests that a rapid equilibrium between allantoic fluid and blood does not occur. Chen et al. (1978) indicated by immunodiffusion that uteroferrin was not detectable in fetal blood or tissues. Moreover, as shown in CHAPTER 3 by 2D gel electrophoresis (NEPHGE), it could not be detected in fetal plasma. The TCA precipitates of fetal serum did reveal some low level precipitation of radioactivity and the

RIA results from CHAPTER 3 showed that low concentrations of uteroferrin were present though much lower than in allantoic fluid (a mean of 2.8 μg/ml in Day 60 fetal blood versus a mean of 333 μg/ml in Day 60 allantoic fluid). Possibly some uteroferrin is transported across a "leaky" allantoic epithelium which Patton (1978) describes as an extension of the fetal hind gut. The transported uteroferrin may then be destroyed as a "foreign" protein in the fetal liver. It is further possible that some of the immunoreactive and TCA precipitates are large fragments and not intact uteroferrin.

The iron distribution in the fetus from ^{59}Fe-uteroferrin injections into allantoic fluid is consistent with the expected metabolism of iron. That is, iron is apparently sequestered in the fetal liver and spleen. Liver tissues are known for their role in iron storage and metabolsim (Moustgaard et al., 1969; Crichton, 1973) and in erythropoeisis which includes hemoglobin synthesis. Iron distribution in the fetus after ^{59}Fe-transferrin injection is very similar to that after supplying ^{59}Fe-uteroferrin.

That transferrin is the acceptor of iron from uteroferrin was well documented. It was shown to co-elute on gel filtration columns with a protein that appeared to accept ^{59}Fe from uteroferrin and was precipitated with specific anti-transferrin antibody. Its presence in allantoic fluid throughout gestation, especially when uteroferrin is present, was demonstrated in CHAPTER 3 by 2D-PAGE studies. In the ^{125}I-transferrin injection experiment, it was clear that the protein could escape the allantoic sac and was in fairly rapid equilibrium with fetal blood, whose specific activity was 34.5% that of the allantoic fluid after 24 hours. In addition, transferrin appeared to be transferred in intact form into the fetal circulation.

Finally, evidence is presented to show that maternal transferrin is not transferred in intact form across the placenta over the 24 h of the experiment. A preliminary experiment in which chorioallantoic membrane was incubated for 48 h with L-[^{3}H] leucine showed no synthesis of transferrin (unpublished). The transferrin in the fetal compartment is likely therefore to be entirely of fetal origin.

CHAPTER 5
MODEL STUDIES OF IRON LOSS AND PROTEIN DEGRADATION

Introduction

Early studies of proteolytic degradation of the iron-binding proteins transferrin, lactoferrin, and ovotransferrin were performed primarily to produce fragments which could be tested for their iron-binding properties. The studies of Williams (1974;1975) and Line et al. (1976) on ovotransferrin and lactoferrin, respectively, were expressly done to produce "half-molecules" in order to study the mechanism of iron-binding and release at each of the two sites without influence from the other site. Studies by Brock et al. (1976) on bovine transferrin and lactoferrin, Bluard-Deconick et al. (1978) on human lactoferrin, and Evans and Williams (1978) on human transferrin were carried out for similar reasons, but also to examine the relative susceptibilities of the apo form of the proteins, the half-iron forms, and the completely iron-saturated forms to proteolytic degradation. Because transferrins seem to consist of two fairly similar iron-binding domains, it has been suggested that the molecules arose via a gene duplication event (Aisen and Listowsky, 1980). Therefore, there have also been attempts made to compare the primary structures of the two halves in order to confirm this hypothesis. Results have indicated that half-molecules can be generated by limited proteolytic digestion and that proteolytic sensitivity is related to the degree of Fe saturation (Evans and Williams, 1978). The two halves each appear capable of binding and releasing Fe but have different compositions and affinities

for the metal (Evans and Williams, 1978; Aisen and Listowsky, 1980). Nevertheless, sufficient structural similarities have been observed to suggest an evolutionary relationship between the two halves. It has not gone unnoticed that uteroferrin has a molecular weight about one-half that of transferrin, possesses a single iron-binding site and shows a number of other similarities to transferrin (Roberts and Bazer, 1980). Possibly uteroferrin represents an evolutionary precursor of the transferrins.

Although proteolysis most probably does not represent a usual mechanism of Fe release from the transferrins, it has not been ruled out in all cases (e.g. placental uptake). Proposed mechanisms have included reduction of the Fe, chelation by molecules having a higher affinity for the metal, protonation (possibly by destabilizing the carbonate-Fe coordination) or a combination of several of these factors (Kojima and Bates, 1978). One purpose of experiments reported in this chapter was to test the sensitivity of uteroferrin to proteolysis before and after Fe removal. Here, we felt the experiments might indicate whether or not proteolysis could be a mechanism for Fe removal. Moreover, since apo-uteroferrin is never recovered from allantoic fluid it seemed likely that the protein is destroyed either after or during Fe removal. Thus, in CHAPTER 3 we showed that major fragments with a molecular weight of about 22,830 and 14,670 could be detected in allantoic fluid after introducing ^{125}I-uteroferrin *in vitro*. Here we tested whether similar fragments could be generated using purified proteases and whether the pattern of uteroferrin degradation resembles in any way that of the transferrins.

The second aim of this chapter was to establish model conditions for transfer of Fe from uteroferrin to transferrin and to test a variety of conditions that might promote Fe release. In particular, we wondered whether the conditions that promote Fe loss from transferrin might also apply to uteroferrin.

Materials and Methods

Materials

The $^{59}FeCl_3$ (7 - 9 μC/mg Fe) was purchased from the Amersham Corporation. All reagents were reagent grade or better. ^{125}I-Con A was a gift from Mr. George Baumbach.

Uteroferrin Purification

Purification methodology is described in CHAPTER 3.

Transferrin Purification

Purification methodology is described in CHAPTER 4.

Apo-Uteroferrin Preparation

Preparation is described in CHAPTER 3.

Apo-Transferrin Preparation

Preparation is described in CHAPTER 4.

^{59}Fe-Uteroferrin Preparation

Preparation is described in CHAPTER 3.

Polyacrylamide Gel Electrophoresis

Single dimension SDS-polyacrylamide gel electrophoresis was performed according to the procedure described in CHAPTER 3 for 15% (w/v) polyacrylamide tube gels. In addition, electrophoresis in 15% (w/v) polyacrylamide slab gels was employed to follow the time courses of proteolytic cleavage of uteroferrin.

Inhibition of Chymotrypsin Activity in Trypsin Preparations

Commercial crystalline preparations of trypsin (Sigma) contain appreciable amounts of chymotrypsin activity (trypsin activity was 262 U/mg protein compared to chymotrypsin activity of 35.6 U/mg protein). To inhibit this protease activity which would give rise to a mixture of complex cleavage products, trypsin was batch treated according to the method of Kostka and Carpenter (1964). In this method 100 mg trypsin in 33 ml of 1 mM $CaCl_2$ was added to 28.4 mg L-1-Tosylamide-2-phenylethyl chloro-methyl ketone (TPCK) previously dissolved in 2.0 ml methanol. This was adjusted to pH 7 with 0.5 N NaOH and allow to stand for 5 hours. The pH was then adjusted to 3.0 with 1 N HCl, the precipitate was removed, and the supernatant dialyzed against cold H_2O (4°C, pH 3). After lypholyzing, the preparation was stored at 4°C. This treatment had no effect on trypsin activity but reduced the chymotrypsin activity by 86.5%.

"Staining" of Gels with ^{125}I-Lectin

In order to determine which polypeptide band or bands in the gels contained carbohydrate, the slab gels were incubated with ^{125}I-concanavalin according to the method of Burridge et al. (1976) as modified by Horst et al. (1980), washed and subjected to autoradiography. Briefly, the method involved first equilibrating the fixed, Coomassie-blue stained gels in three changes of Tris-buffered saline (0.05 M, 0.15 M, pH 7.4). The gels were then placed in a seal-a-meal plastic bag and buffer drained out. The ^{125}I-Con A (10^6 cpm/ml), containing hemoglobin (1 mg/ml), was added to the bag and sealed. The control gel contained ^{125}I-Con A (10^6 cpm/ml) and α-methylmannose (0.5 M). The sealed bags were placed on a rotating platform shaker

overnight at room temperature. Later, the bags were opened, the gels removed, and washed two times per day with the above buffer until the wash counts were near background levels. The gels were dried and autoradiographs prepared using Kodak XRP-5 x-ray film.

Periodic Acid-Schiff Stain

Here the gels were equilibrated with 7% (v/v) acetic acid: 40% (v/v) ethanol in order to remove SDS and then rinsed in 7% (v/v) acetic acid. Following this, the gels were consecutively soaked in 0.5% (v/v) acetic acid containing 0.5% (w/v) periodic acid for 2 h, 0.5% (w/v) sodium arsenite for 30 - 60 min, and 0.1% (w/v) sodium arsenite (with two changes). After soaking in 0.5% (v/v) acetic acid for 20 min they were placed in Schiff's reagent overnight. After washing they were destained using two changes of 0.1% (w/v) sodium disulfite in 0.1 N HCl for 30 min and washed in water.

Proteolytic Digestion of Uteroferrin by Trypsin

Uteroferrin (usually 1 mg/ml) was incubated in buffer (0.05 M Tris-HCl, 0.02 M $CaCl_2$, pH 8.0) at 37°C with TPCK-treated trypsin. The enzyme/substrate ratios (w/w) were 1/10, 1/25, 1/50, and 1/100. The incubations were for various time periods up to 96 hours. Trypsin in the samples was inhibited with 5 units of Trasylol (FBS Pharmaceuticals, N.Y.) per µg trypsin. In some experiments, the incubations were frozen immediately without addition of Trasylol.

Apo-uteroferrin (0.5 mg/ml) was treated similarly except a single enzyme/substrate ratio was used (1/50 w/w).

Chymotrypsin Digestion of Uteroferrin

Uteroferrin (1 mg/ml) was incubated in buffer (0.05 M Tris-HCl, 0.02 M $CaCl_2$, pH 8.0) at 37°C with chymotrypsin. The concentrations

of enzyme and substrate were in ratios (w/w) of 1/10, 1/25, 1/50, and 1/100. After incubation for time periods up to 96 h at 37°C, the samples were frozen immediately and stored at -20°C until analyzed.

Procedure for the Study of Iron Loss from ^{59}Fe-Uteroferrin

Chelators and ascorbic acid solutions were prepared fresh prior to use and brought to pH 6.8 before addition to each reaction (final concentration 1 mM). Reactions were buffered with 0.025 M MOPS, pH 6.8. All incubations were held at 37°C in tubes sealed with at least five layers of parafilm. Total reaction volumes were 0.5 ml. Each reaction contained 110 µg ^{59}Fe-uteroferrin and either 115 µg, 106 µg, or 107 µg of apo-transferrin, transferrin, or ferritin, respectively. All incubations were for 64 h, at which time they were quickly frozen and stored at -20°C until analyzed.

Samples were quickly thawed in warm water, the radioactivity of each determined, and the salt concentration adjusted to 0.4 M in NaCl by adding solid NaCl. They were then loaded onto Sephadex G-100 columns (1.5 x 60 cm), equilibrated and eluted with barbital-saline buffer (0.02 M, 0.4 M, pH 7.8), to separate the reactant. Fractions (1.1 ml) were collected and the radioactivity in each determined. Control experiments using ^{59}Fe-uteroferrin incubated for 48 h and 96 h at pH 6.5 gave a recovery of between 88% and 77%, respectively, of ^{59}Fe-uteroferrin. Approximately 9 - 10% of the ^{59}Fe appeared in the salt volume.

Procedure for the Study of Reactions Involving ^{59}Fe-Uteroferrin, Apo-Transferrin, and Ascorbic Acid

Reactions were carried out in a manner similar to that described above. The ^{59}Fe-uteroferrin and apo-transferrin were added to reactions

at 94 µg and 98 µg, respectively. Ascorbic acid was used at three concentrations; 0.02 mM, 0.1 mM, and 1 mM, each at three different incubation times (24, 48, and 72 h). Additional controls included ^{59}Fe-uteroferrin incubated in buffer alone and in the presence of citrate, ascorbate, and ATP (all at 1 mM). Sephacryl S-200 columns (1.5 x 96 cm) were equilibrated and eluted as above.

An additional experiment was performed with ascorbic acid at 0.02 mM and 1 mM essentially as described above. However, the incubation was performed in sealed glass vials containing an argon atmosphere for 96 h in order to preclude the spontaneous oxidation of ascorbic acid to dehydroascorbate.

Effect of pH on Iron Release from ^{59}Fe-Uteroferrin

^{59}Fe-uteroferrin (94 µg) was incubated in 0.025 M buffer solutions (KCl-HCl, Phthalate-HCl, Acetate, MES, MOPS, and Tris-HCl) in order to cover pH values between 2 and 8. Each incubation was set up in duplicate, one of which contained 1 mM ATP. All were incubated at 37°C for 48 h in tubes sealed with five layers of parafilm. After 48 h all were frozen at -20°C until analyzed. Samples were analyzed by gel filtration on Sephacryl S-200 as described above.

Results

Degradation of Purified Uteroferrin by Trypsin

These experiments were performed to examine the degradation of uteroferrin by a known protease as a possible model for proteolytic cleavage *in vivo*. The intent was to compare results with previous experiments (CHAPTER 3) in which proteolytic cleavage of uteroferrin was followed in allantoic fluid *in vitro*.

When uteroferrin was incubated with TPCK-treated trypsin in various enzyme/substrate ratios its acid phosphatase activity was shown to be lost in a time-dependent manner (Figure 5-1) although at the low ratio (1/100) there was a lag before loss of activity was noted. Subsequent electrophoretic analysis of the digests on 15% (w/v) polyacrylamide gels after treatment with β-mercaptoethanol and SDS revealed a constant pattern of proteolytic cleavage. Two major size classes of fragments (A and B) were evident (Figure 5-2). Upon close examination, each class appeared to be composed of two closely migrating bands, one usually staining more heavily than the other. With increasing time, the uteroferrin band gradually decreased in staining intensity. At a ratio of trypsin to uteroferrin of 1:50, most of the uteroferrin had been cleaved by 3 h even though most of the phosphatase activity had not been lost. Bands A and B also began to decrease after 6 hours. Bands A1 and A2 had estimated molecular weights of 20,750 and 19,750, respectively, with A2 always greater in amount than A1. Bands B1 and B2, which migrated very close together, had molecular weights of about 14,500.

The digest was also examined to determine which fragment(s) contained carbohydrate since uteroferrin is a glycoprotein (Chen et al., 1973). Two techniques, ^{125}I-lectin binding (here ^{125}I-Con A) and PAS staining were employed (Figures 5-3A and B). Both indicated that only bands A1 and A2 contained carbohydrate. Bands B1 and B2 did not bind Con A nor stain by the PAS procedure.

Electrophoretic analysis of the trypsin digests in the presence of SDS, but in the absence of β-mercaptoethanol, revealed what appeared to be only minor degradation at 3 h and 30 h although the uteroferrin band was slightly decreased in intensity. The same samples treated

Figure 5-1. Specific Activity of Uteroferrin after Incubation with Trypsin as Long as 96 h at Different Trypsin/Uf Ratios

Uteroferrin (1 mg/ml) was incubated in Tris-HCl buffer (0.05 M, pH 8.0) containing 0.02 M $CaCl_2$ at 37°C with TPCK-treated trypsin up to 96 hours. The trypsin/Uf ratios (w/w) were 1/10, 1/25, 1/50, and 1/100. The acid phosphatase activity of each digest was then determined using the standard assay. The specific activity is μmoles p-NP released/min/mg protein.

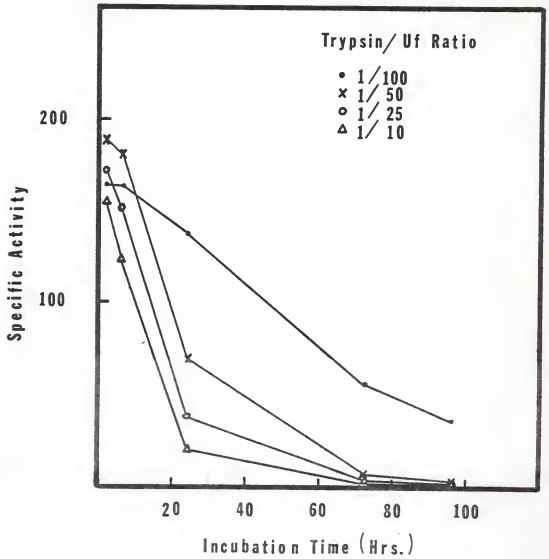

Trypsin/ Uf Ratio
• 1/100
× 1/ 50
○ 1/ 25
△ 1/ 10
200
100
Specific Activity
20
40
60
80
100
Incubation Time (Hrs.)

Figure 5-2. One Dimensional SDS-Polyacrylamide Gel Electrophoresis of Uteroferrin After Incubation with Trypsin as Long as 96 h

Uteroferrin (1 mg/ml) was incubated in Tris-HCl buffer (0.05 M, pH 8.0) containing 0.02 M $CaCl_2$ at 37°C with TPCK-treated trypsin up to 96 h in an enzyme/substrate ratio of 1/50 (w/w). At various times aliquots (1 ml) were removed and 5 units of Trasylol per µg trypsin added and the samples were frozen at -20°C. Later, the samples were treated with 2% (w/v) SDS and 10% (v/v) β-ME and subjected to one dimensional 15% (w/v) PAGE. To each tube 25 µg protein was loaded. After electrophoresis the gels were stained with Coomassie blue.

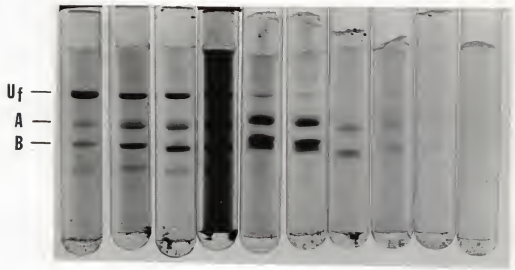

Uf
A
B
.08
.5
1
2
3
6
30
48
72
96
HOURS

Figure 5-3A. One Dimensional SDS-Polyacrylamide Gel Electrophoresis of a Trypsin Digest of Uteroferrin and Apo-uteroferrin "Stained" with ^{125}I-Con A and Coomassie Blue and a Uteroferrin Digest Stained with PAS and Coomassie Blue

Uteroferrin (1 mg/ml) and apo-uteroferrin (0.5 mg/ml) in Tris-HCl buffer (0.05 M, pH 8.0) containing 0.02 M $CaCl_2$ were treated with TPCK-trypsin in a enzyme/substrate ratio (w/w) of 1/50. These were subjected to one dimensional slab gel electrophoresis as previously described in the text and stained with Coomassie blue. The slab gels were cut into sections, placed in seal-a-meal plastic bags, and treated with ^{125}I-Con A (10^6 cpm/ml). The control had α-methylmannose (0.5 M) added. After 24 h the gels were washed to background levels, dried, and autoradiographs prepared. The upper photos (A1 and A2) are the Coomassie stained gels and the lower photos are the autoradiographs of the dried ^{125}I-Con A treated gel (B1 with and B2 without 0.5 M α MM).

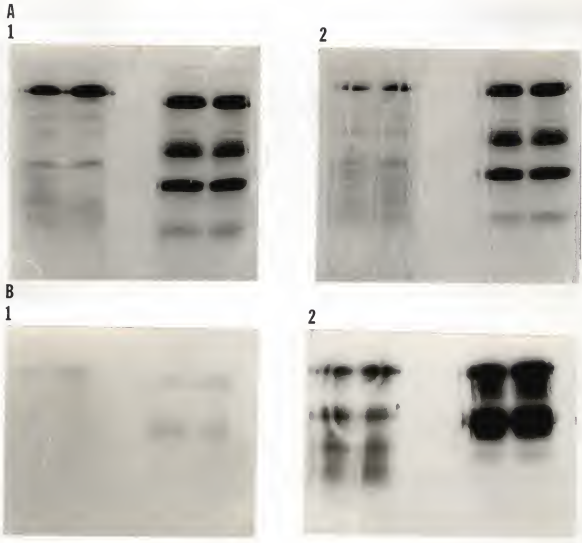
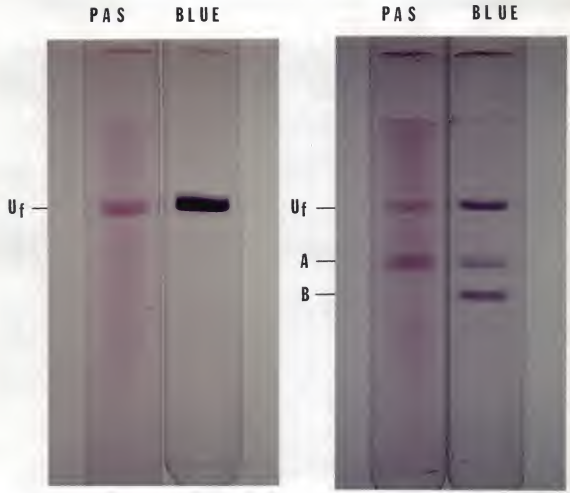

A
1
2
B
1
2

Figure 5-3B. One Dimensional SDS-Polyacrylamide Gel Electrophoresis of a Trypsin Digest of Uteroferrin and Apo-uteroferrin "Stained" with ^{125}I-Con A and Coomassie Blue and a Uteroferrin Digest Stained with PAS and Coomassie Blue

Uteroferrin (1 mg/ml) in Tris-HCl buffer (0.05 M, pH 8.0) containing 0.02 M $CaCl_2$ was treated with trypsin as previously described. The control and 1 h sample were subjected to one dimensional 15% (w/v) polyacrylamide gel electrophoresis in tubes as previously described. On each gel 50 µg protein was loaded. These were stained both with Coomassie blue and by PAS. The left photo represents the control Uf and the right photo represents the trypsin treated Uf.


PAS
BLUE
Uf
PAS
BLUE
Uf
A
B

with β-mercaptoethanol, however, indicated that considerable polypeptide cleavage had occurred (Figure 5-4).

Degradation of Apo-Uteroferrin by Trypsin

Previous studies by Brock et al. (1976) indicated that bovine transferrin was cleaved by trypsin to yield two fragments and that apo-transferrin was more susceptible to cleavage than the Fe-containing form yielding as product only a small amount of one of the two fragments. In this section the susceptibility of apo-uteroferrin to digestion by trypsin was examined.

Apo-uteroferrin was incubated with TPCK-treated trypsin in an enzyme substrate ratio of 1/50 (w/w) for as long as 48 hours. Electrophoretic analysis after treatment with β-mercaptoethanol and SDS on 15% (w/v) polyacrylamide gels revealed a progressive proteolysis of the apo-uteroferrin with only a minor accumulation of bands similar in molecular weight to A and B in the previous section (Figure 5-5). These bands comprised only a small proportion of the total Coomassie-blue staining material. Nearly all of the apo-uteroferrin and the other peptides had been degraded by 3 hours. These results clearly indicate that apo-uteroferrin is degraded at a faster rate than the Fe form (Figure 5-6). Moreover, polypeptides of low molecular weight do not appear as stable intermediates.

Degradation of Uteroferrin by Chymotrypsin

In this section we examined whether a second protease would give a similar pattern of uteroferrin cleavage and generate similar fragments.

Uteroferrin (1 mg/ml) was therefore incubated with chymotrypsin at an enzyme/substrate ratio of 1/50 (w/w). After treatment with

Figure 5-4. One Dimensional SDS-Polyacrylamide Gel Electrophoresis With and Without β-Mercaptoethanol of Uteroferrin After Incubation with Trypsin for 3 h and 30 h

Uteroferrin (1 mg/ml) in buffer incubated with trypsin as previously described for 3 h and 30 h was subjected to 15% (w/v) polyacrylamide gel electrophoresis in one dimension. The samples were either treated with or without 10% (w/v) β-ME before loading. Twenty-five micrograms of the β-ME treated sample were loaded on each gel and 50 μg of the nontreated sample were loaded on the other gels. After electrophoresis the gels were stained with Coomassie blue. (a = treatment with 2% (w/v) SDS; b = treatment with 2% (w/v) SDS and 10% (v/v) βME)

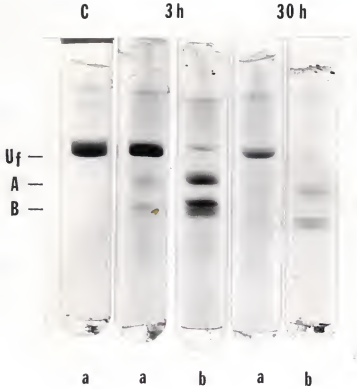

C
3 h
30 h
Uf
A
B
a
a
b
a
b

Figure 5-5. One Dimensional SDS-Polyacrylamide Gel Electrophoresis of Apo-uteroferrin After Incubation with Trypsin as Long as 24 h

Apo-uteroferrin (0.5 mg/ml) in Tris-HCl buffer (0.05 M, pH 8.0) containing 0.02 M $CaCl_2$ was incubated with trypsin as previously described in an enzyme/substrate ratio (w/w) of 1/50 as long as 24 h. Samples were subjected to 15% (w/v) polyacrylamide gel electrophoresis in tubes as previously described. One hundred micrograms were loaded on each gel. After electrophoresis the gels were stained with Coomassie blue.

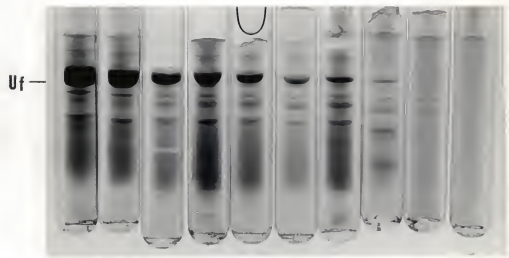
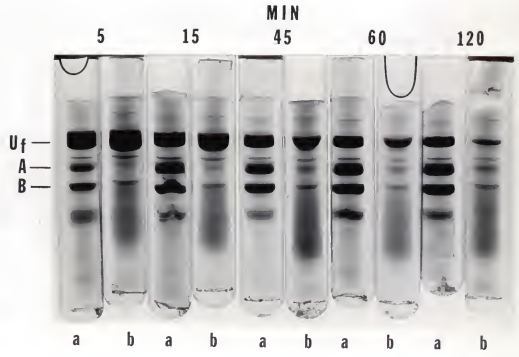

Uf
.08 .25 .5 .75 1 1.5 2 3 6 24
HOURS

Figure 5-6. Comparison of One Dimensional SDS-Polyacrylamide Gel Electrophoresis of Uteroferrin and Apo-uteroferrin After Treatment with Trypsin from 5 to 120 min

Digests of uteroferrin and apo-uteroferrin as described earlier were subjected to 15% (w/v) polyacrylamide gel electrophoresis and treated as described in the text. One hundred micrograms were loaded on each gel and after electrophoresis stained with Coomassie blue. (a = treatment with 2% (w/v) SDS and 10% (v/v) βME; b = treatment with 2% (w/v) SDS)


MIN
5
15
45
60
120
Uf
A
B
a
b
a
b
a
b
a
b
a
b

β-mercaptoethanol and SDS, the samples were again analyzed by electrophoresis on 15% (w/v) polyacrylamide gels. The pattern of degradation (Figure 5-7), namely the appearance of two major size fragments (A and B) was very similar to that observed with trypsin. The A and B bands were each composed of two components. The faster migrating group (B) contained polypeptides with very similar mobilities. Nevertheless at low loading concentrations they were always distinguishable. Bands A1 and A2 did not stain heavily with Coomassie-blue. The estimated molecular weights of these bands were A1, 19,060, A2, 18,280, and B, 14,500.

When chymotrypsin digests were examined by electrophoretic analysis in the absence of β-mercaptoethanol, the pattern produced was different than with the reducing agent (Figure 5-8). The second major fragment (B) was not seen. In addition, a third A band appeared with a slightly higher estimated molecular weight than A1 or A2. In this experiment with mercaptoethanol a third A band was also found.

Reactions Between ^{59}Fe-Uteroferrin and Apo-Transferrin, Transferrin, and Ferritin

Carver and Frieden (1978) have shown that certain phosphate compounds, e.g. ATP and PP_i, can mediate the release of transferrin iron while Harris (1978), using small molecular weight chelators, e. g. citrate, has indicated these may play a role in mediating iron transfer between ferritin and transferrin. Since uteroferrin transfers iron to transferrin *in vivo* (CHAPTER 4), we examined whether this transfer could occur in simple buffer solutions and whether it was promoted by various low molecular weight compounds which are likely to interact with the protein or its iron.

In one group of experiments ^{59}Fe-uteroferrin was incubated for 64 h with apo-transferrin in the presence of 1 mM ATP, citrate,

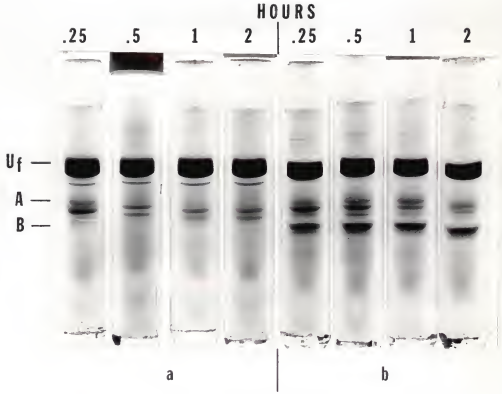
Figure 5-7. One Dimensional SDS-Polyacrylamide Gel Electrophoresis of Uteroferrin After Incubation with Chymotrypsin as Long as 48 h

Uteroferrin (1 mg/ml) in Tris-HCl buffer (0.05 M, pH 8.0) containing 0.02 M $CaCl_2$ was incubated with chymotrypsin in an enzyme/substrate ratio (w/w) of 1/50 as long as 48 h. At various times aliquots (1 ml) were removed and frozen at -20°C. Later, the samples were subjected to 15% (w/v) polyacrylamide gel electrophoresis in tubes as previously described after treatment with 2% (w/v) SDS) and 10% (v/v) β-ME. The gels were stained with Coomassie blue after electrophoresis.


Uf
A
B
.08 .25 .5 .75 1 3 6 24 48
HOURS

Figure 5-8. One Dimensional SDS-Polyacrylamide Gel Electrophoresis of a Chymotrypsin Digest of Uteroferrin Treated Either With or Without β-Mercaptoethanol

The chymotrypsin digest of uteroferrin described above was subjected to 15% (w/v) polyacrylamide gel electrophoresis after treatment with or without β-mercaptoethanol. Samples of 100 μg were loaded on each gel and after electrophoresis stained with Coomassie blue. (a = treatment with 2% (w/v) SDS; b = treatment with 10% (v/v) βME)


HOURS
.25
.5
1
2
.25
.5
1
2
Uf
A
B
a
b

ascorbic acid, sodium pyrophosphate and in a combination of these with ascorbic acid. The results of a typical experiment as analyzed by gel filtration are presented in Figure 5-9. Apo-transferrin alone had only a limited ability to remove Fe from uteroferrin when the two proteins were incubated together in buffer alone. However, all of the low molecular weight compounds promoted transfer of Fe to transferrin (Table 5-I). This was especially true with ascorbic acid which mediated transfer of almost 85% of the Fe. Only small amounts of ^{59}Fe were found in the salt volume where ascorbate itself and putative chelates are likely to be recovered. Ascorbate, in combination with the other agents, released Fe in amounts comparable to ascorbate alone (i.e. there was no apparent synergism). Neither citrate nor ascorbate were particularly effective agents of Fe release if apo-transferrin was not present, releasing between 25 - 30% of the iron by 72 hours.

In a second group of experiments, purified porcine serum transferrin (presumed to be partially saturated with Fe) was used as above. Again, Fe was released from uteroferrin and largely accepted by the transferrin. However, transfer was generally less effective (Table 5-II). More ^{59}Fe appears in the salt volume than previously shown (see Table 5-I) and is most likely associated with chelator. The reasons for poor recovery of ^{59}Fe in several experiments are unclear.

In a third group of experiments conducted as above, ferritin was used as the Fe acceptor to examine the possibility that uteroferrin could also release Fe to ferritin as it does to transferrin (Table 5-III). Very poor recoveries (as low as 25%) of ^{59}Fe were achieved in those reactions involving ascorbate. However, it was evident that even in

Figure 5-9. Sephadex G-100 Chromatography of Incubation Mixtures Containing ^{59}Fe-uteroferrin, Apo-transferrin and 1 mM ATP, Citrate, Ascorbate or Buffer

Uteroferrin (110 µg) labelled with ^{59}Fe was incubated with apo-transferrin (115 µg) and either buffer alone (0.025 M MOPS, pH 6.8) or 1 mM ATP, citrate, or ascorbate for 64 hours. The samples (0.5 ml) were loaded on Sephadex G-100 columns (1.5 x 60 cm), equilibrated and eluted with barbital-saline buffer (0.02 M, 0.4 M, pH 7.8), and the radioactivity of the fractions (1.1 ml) determined.

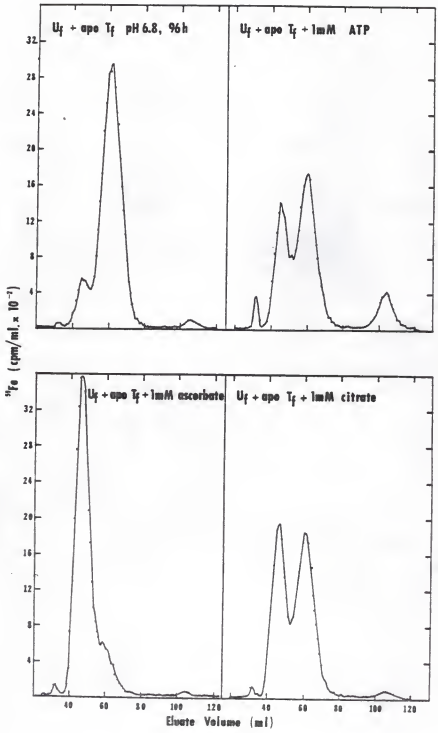

U_f + apo T_f pH 6.8, 96h
U_f + apo T_f + 1mM ATP
U_f + apo T_f + 1mM ascorbate
U_f + apo T_f + 1mM citrate
^{59}Fe (cpm/ml x 10^{-3})
Elute Volume (ml)

Table 5-1

Effect of Citrate, Ascorbate, ATP, Pyrophosphate Alone and in Combination with Ascorbate on Fe Transfer from ^{59}Fe-uteroferrin to Apo-transferrin

Mediating Agent(1)	Iron Distribution at the End of the Experiment (Expressed as % of ^{59}Fe Recovered)					
	% ^{59}Fe in Uf	% ^{59}Fe in Tf	% ^{59}Fe in Void	% ^{59}Fe in Chelate	% ^{59}Fe Recovered	% ^{59}Fe Lost from Uf
None	86.7	10.9	0.4	2.0	98.9	13.3
Citrate	55.1	41.8	0.9	2.2	93.9	44.9
Ascorbate	17.8	79.8	1.1	1.2	95.9	82.2
ATP	52.7	32.7	2.9	11.6	90.6	47.3
PP_i	67.8	27.5	0.5	4.2	93.4	32.2
Citrate/Ascorbate	9.3	88.1	0.9	1.6	93.9	90.7
ATP/Ascorbate	26.2	56.4	8.1	9.3	87.5	73.8
PP_i/Ascorbate	15.1	72.2	4.1	8.5	91.2	84.9
Citrate(2)	70.5	Not Present	0.3	29.2	86.1	29.5
Ascorbate(2)	73.0	Not Present	0.2	26.7	76.0	27.0
ATP(2)	94.0	Not Present	1.0	4.9	81.7	6.0

(1) All reducing and chelating agents were 1 mM
(2) These were incubated for 72 h and all others for 64 h

Table 5-II

Effect of Citrate, Ascorbate, ATP, Pyrophosphate Alone and in Combination with Ascorbate on Fe Transfer from ^{59}Fe-uteroferrin to Transferrin(1)

Mediating Agent(2)	Iron Distribution at the End of the Experiment (Expressed as % of ^{59}Fe Recovered)					
	% ^{59}Fe in Uf	% ^{59}Fe in Tf	% ^{59}Fe in Void	% ^{59}Fe in Chelate	% ^{59}Fe Recovered	% ^{59}Fe Lost from Uf
None	89.5	4.0	1.1	5.4	89.3	10.5
Citrate	60.9	29.0	1.8	8.3	88.4	39.1
Ascorbate	44.7	40.5	3.1	11.8	86.8	55.3
ATP	9.3	8.0	15.1	67.6	62.8	90.7
PP_i	74.3	12.1	1.0	12.6	85.5	25.7
Citrate/Ascorbate	19.6	49.0	4.2	27.2	94.9	80.4
ATP/Ascorbate	46.8	30.4	8.0	14.8	73.2	53.2
PP_i/Ascorbate	18.6	16.1	17.9	47.3	62.8	81.4

(1) Incubation time was 64 h
(2) All reducing and chalating agents were 1 mM

Table 5-III

Effect of Citrate, Ascorbate, ATP, Pyrophosphate Alone and in Combination With Ascorbate on Fe Transfer from ^{59}Fe-uteroferrin to Ferritin(1)

Mediating Agent(2)	Iron Distribution at the End of the Experiment (Expressed as % of ^{59}Fe Recovered)				
	% ^{59}Fe in Uf	% ^{59}Fe in Ferritin	% ^{59}Fe in Chelate	% ^{59}Fe Recovered	% ^{59}Fe Lost from Uf
None	81.8	14.1	4.1	90.3	18.2
Citrate	53.3	41.9	4.7	78.9	46.7
Ascorbate	80.2	7.8	11.9	28.6	19.8
ATP	50.9	33.9	15.2	75.5	49.1
PP_i	70.4	21.3	8.3	92.2	29.6
Citrate/Ascorbate	37.9	6.5	55.7	51.8	62.1
ATP/Ascorbate	65.2	13.1	21.7	28.1	34.8
PP_i/Ascorbate	53.2	7.2	39.6	25.6	46.8

(1) Incubation time was 64 h
(2) All reducing and chelating agents were 1 mM

these experiments Fe could be transferred to this iron storage protein provided a low molecular weight chelator is present.

Reactions Between ^{59}Fe-Uteroferrin and Apo-Transferrin Mediated by Ascorbic Acid

In the previous experiments, ascorbate (1 mM) was shown to be an effective mediator of Fe-transfer from uteroferrin to transferrin. In this section different concentrations of ascorbate were employed over a range that spanned and exceeded those found in allantoic fluid. ^{59}Fe-uteroferrin and apo-transferrin were incubated with ascorbic acid (0.02 mM, 0.1 mM and 1 mM) for 24, 48 and 72 h (Table 5-IV). At 1.0 mM most of the Fe was released and transferred to apo-transferrin within the first 24 h with little lost thereafter. With 0.1 mM, equilibrium was established more slowly. At concentrations of 0.02 mM, 0.1 mM, and 1.0 mM ascorbic acid, 32.4%, 55.6%, and 84.3% of the ^{59}Fe, respectively, had been lost from uteroferrin by 72 hours. The amount of low molecular weight ^{59}Fe (in the salt volume) remained constant at all times.

An experiment similar to the above, with ascorbic acid at concentrations of 0.02 mM and 1 mM, was performed but in sealed glass tubes under argon. Over an incubation period of 96 h, 65.4% of the Fe was lost at 1 mM ascorbate (i.e. slightly less than that in the previous experiment) while with 0.02 mM ascorbate the value was 47.8% Fe lost (i.e. a somewhat higher amount than that in the previous experiment).

Thus, under slightly acid conditions, at a pH similar to that of allantoic fluid (6.8), with low ascorbic acid concentrations, Fe can be transferred from uteroferrin to apo-transferrin. Transfer in the absence of ascorbate is slow.

Table 5-IV

Effect of Different Ascorbate Concentrations on Fe Transfer From ^{59}-uteroferrin to Apo-transferrin

Ascorbic Acid (mM)	Incubation Time (h)	Iron Distribution Expressed as % of ^{59}Fe Recovered		
		$\%^{59}$Fe in Uf	$\%^{59}$Fe in apo Tf	$\%^{59}$Fe lost from Uf
0.02	24	66.5	27.4	33.5
	48	61.7	34.3	38.3
	72	67.6	28.0	32.4
0.1	24	50.4	38.9	49.6
	48	43.6	48.9	56.4
	72	44.4	50.7	55.6
1.0	24	27.0	59.9	73.0
	48	19.5	63.6	80.5
	72	15.7	68.7	84.3
None	72	80.7	15.1	19.3

Effect of pH on Fe Release

Clearly a number of chelators can mediate a transfer of Fe from uteroferrin to transferrin. Loss of Fe from transferrin in the presence of a suitable reactant has been shown to be sensitive to changes (lowering) in pH (Morgan, 1977; Carver and Frieden, 1978). In this section, the effect of pH over the range 2-8 on the release of Fe from uteroferrin in the presence and absence of ATP (1 mM) is discussed. The method employed a 48 h incubation of ^{59}Fe-uteroferrin with or without ATP in the various buffer systems after which gel filtration was performed to separate the protein bound and free ^{59}Fe. In Figure 5-10 the amount of ^{59}Fe remaining in the ^{59}Fe-uteroferrin after incubation is plotted against pH. Clearly there was no loss of ^{59}Fe from uteroferrin until the pH fell below 4. At pH 2.0 all of the ^{59}Fe was lost. ^{59}Fe-uteroferrin was completely stable between pH 5 and 8. The presence of ATP in the reaction mixture did not appear to influence Fe release.

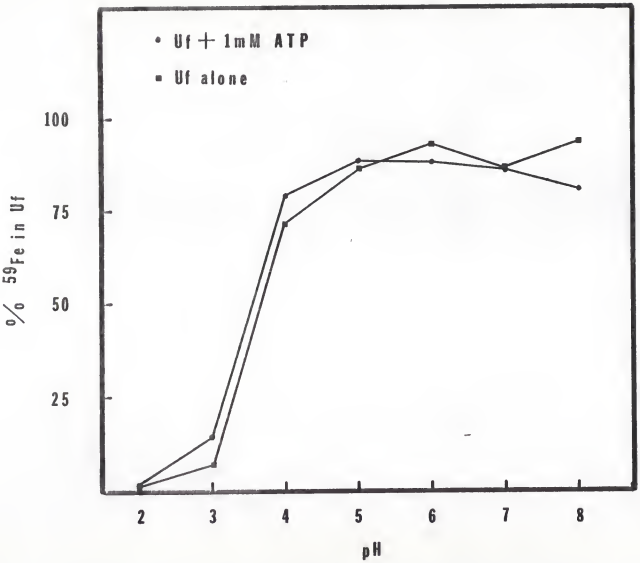
Discussion

Polypeptide Cleavage

It has previously been reported (Brock et al., 1978) that Fe-saturated transferrins are fairly resistant to proteolysis. Evans and Williams (1978) have, however, demonstrated from studies using SDS-PAGE with and without β-mercaptoethanol that human transferrin undergoes some internal cleavage and the resulting fragments are held together by disulfide bridges. The iron-binding properties were unimpaired. Earlier experiments reported in CHAPTER 3 indicated that uteroferrin was degraded slowly in allantoic fluid *in vitro* and that apoprotein (Fe-free) was highly sensitive to proteolysis. Degradation

Figure 5-10. Loss of Fe from ^{59}Fe-uteroferrin With and Without ATP Present as a Function of pH

Uteroferrin (94 µg) labelled with ^{59}Fe was incubated in duplicate in various 0.025 M buffers to cover the pH range between 2 and 8. One of the duplicates contained 1 mM ATP. All were incubated at 37°C in tubes sealed with five layers of parafilm for 48 h and then frozen at -20°C until analyzed. All analysis was performed as described in the text by gel filtration on Sephacryl S-200. The fractions (1.1 ml) were determined for radioactivity and the percent ^{59}Fe present in Uf calculated.


• Uf + 1mM ATP
▪ Uf alone
100
75
50
25
% 59Fe in Uf
2
3
4
5
6
7
8
pH

of ^{125}I-uteroferrin in Day 60 allantoic fluid (CHAPTER 3) gave major fragments of 22,830 and 14,670 as estimated by SDS-PAGE. In the present chapter, the sensitivity of uteroferrin to two model proteases, trypsin, and chymotrypsin was tested. Even though these have different epecificities, they each cleaved Fe-uteroferrin in a similar manner, in the sense that two major polypeptide fragments (A and B, M_r 18,000 - 20,000 and 14,500) were generated. Williams (1974) observed a similar finding with ovotransferrin. Here, the fragments could be detected by SDS-PAGE only if disulfide bonds were first reduced by β-mercapto-ethanol. Since a fairly similar pattern of fragmentation was observed when uteroferrin was incubated in allantoic fluid *in vitro*, it is tempting to suggest that the protein possesses a loop of polypeptide which is readily accessible to protease attack. A second conclusion that can be drawn from the experiments is that the clipped polypeptide remains fully active as a phosphatase and presumably, therefore, still binds its iron since Schlosnagle et al. (1976) have shown that phosphatase activity is correlated with iron content. For example, between 3 and 6 h, using a ratio of trypsin to uteroferrin of 1 to 50, nearly all of the uteroferrin has been internally cleaved, yet the phosphatase activity had only begun to decline. Fragments A and B are clearly held together by disulfide bonds and polypeptide cleavage at the sensitive region does not influence phosphatase activity.

It is interesting to note that the larger fragments (A1 and A2) generated by trypsin appear to contribute the carbohydrate-containing portion of the molecule. Evans and Williams (1978), in trypsin studies on human transferrin, have shown that the C-terminal fragment, the larger of the two generated, contained both the carbohydrate chains

of transferrin. Brock et al. (1977), in trypsin studies of bovine transferrin, also showed that the larger of two fragments generated contained the carbohydrate. The fragments A1 and A2 differ by about 1,000 in molecular weight and this could be due to variability in carbohydrate content among different uteroferrin molecules. Since the additive molecular weights of A and B are about equal to that of intact uteroferrin, it seems likely that one fragment represents the N-terminal, the other the C-terminal. However, it is not clear which of the two polypeptides, A or B, possesses the iron-binding site. It is possible that iron-binding is a function of both fragments together. The fact that it contains carbohydrate should allow it to be separated easily from B by lectin affinity chromatography. This would allow us to test the iron-binding properties of each polypeptide separately and to establish which originates from the N-terminal and which from the C-terminal end.

When apo-uteroferrin was incubated with trypsin it was rapidly hydrolyzed to small polypeptides. Fragments A and B were not detected. Similar results were observed when apo-uteroferrin was incubated in allantoic fluid. Apo-transferrin, apo-lactoferrin, and apo-ovotransferrin also showed increased sensitivity to proteases (Williams; 1974; Brock et al., 1977; Evans and Williams, 1978). It is not uncommon for removal of prosthetic groups to destabilize proteins such that they are more easily hydrolyzed by proteolytic enzymes *in vitro* and destroyed more quickly *in vivo* (Goldberg and Dice, 1974). Presumably folding is less precise in the absence of the ligand.

Removal of Iron

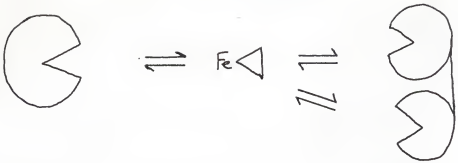
The conditions which influence the release of Fe from transferrin and lactoferrin have been the subject of many investigations (Graham and Bates, 1977; Egyed, 1977; Williams and Evans, 1977; Carver and Frieden, 1978; Harris, 1978; Morgan, 1979; Kojina and Bates, 1979; Konopka et al., 1980; Mazurier and Spik, 1980). Several types of factors appeared to influence loss of iron. These include chelation by high affinity acceptors, reduction of the Fe^{3+} to Fe^{2+} so that it no longer binds to the protein and protonation with regard to the latter. Transferrin iron binding is highly sensitive to pH and is lost rapidly below pH 5.0 (Carver and Frieden, 1978). Uteroferrin, like lactoferrin, seems to have an Fe-binding site which is relatively stable to low pH (Mazurier and Spik, 1980).

Carver and Frieden (1978) have emphasized that a key chelator of transferrin Fe during its removal might be one containing the pyrophosphate structure. Konopka et al. (1980) have confirmed that pyrophosphate releases Fe and mediates the transfer of the metal to ferritin. Interestingly, both pyrophosphate and ATP will catalyze the transfer of Fe from uteroferrin to transferrin. The fact that both of these compounds are substrates for uteroferrin phosphatase activity (Schlosnagle et al., 1976) suggests that they can bind to the active site which is presumed to be close to the Fe whose presence is required for enzymatic activity (Schlosnagle et al., 1976). However, it should be emphasized that neither transferrin or lactoferrin are phosphatases. Nevertheless, the enzymatic activity of uteroferrin may merely be a reflection of the ability of compounds with a pyrophosphate structure to approach the Fe binding site with the metal coordinated at this site serving as a electrophilic catalyst in the

hydrolysis of the pyrophosphate bond. This implies that the phosphatase activity of uteroferrin may be incidental to its true role. We have proposed earlier that the major function of uteroferrin is in Fe metabolism and not in phosphate cleavage.

Although pyrophosphate, ATP, and citrate can mediate the Fe transfer from uteroferrin to transferrin, ascorbate is possibly the best candidate for this role in allantoic fluid. Thus, ascorbate has been detected in both uterine secretions and allantoic fluid (Zavy, 1979; F. W. Bazer, unpublished) in concentrations (0.1 mM and greater) that are effective in Fe transfer. The question arises as to whether it accomplishes Fe removal from uteroferrin by reduction or by chelation. The former seems unlikely since iron removal from uteroferrin does not occur if the protein and ascorbate (1 mM) are incubated together without transferrin. Only at fairly high concentrations (> 0.1 M) do ascorbate and mercaptoethanol (another mild reducing agent) release ferrous iron from uteroferrin at a measurable rate (Schlosnagle et al., 1976). Moreover, transferrin itself binds Fe^{2+} poorly if at all (Gaber and Aisen, 1970; Bates et al., 1973) suggesting that the Fe released from uteroferrin is in Fe^{3+} form.

A general mechanism for Fe release, therefore, is that the low molecular weight chelator (whether ascorbate, ATP, pyrophosphate, or citrate) can compete with uteroferrin for its bound Fe. The association constant of uteroferrin for Fe is presumably much higher than that of the chelator since little low molecular weight Fe can be detected. However, in the presence of apo-transferrin, the following equilibration begins to be established:



with the low molecular weight chelator acting as a mobile carrier which can approach the iron-binding sites on both proteins.

We must assume from our results that the Ka of transferrin for iron is higher than that of uteroferrin for the metal since the overall equilibrium appears to favor transferrin. Since the Ka of the different iron binding sites of transferrin is still controversial, we have not attempted to estimate the binding constant of Fe for uteroferrin from these results.

CHAPTER 6
CONCLUSIONS

1. Uteroferrin, the progesterone-induced purple glycoprotein of the porcine uterus has a molecular weight of about 35,000, binds one atom of iron (most probably in the Fe^{3+} form), and has a molar extinction coefficient at 545 nm of about 3.1×10^{-3} M^{-1} when this is expressed on a dry weight basis.

2. Uteroferrin, which is synthesized by the surface and glandular epithelium of the pregnant uterus, accumulates in allantoic fluid of the conceptus. The average uteroferrin content of the allantoic fluid from a single Day 60 pregnant uterus as determined by RIA is between 830 and 1400 mg.

3. When sterile allantoic fluid is incubated in vitro at 37°C, there is a time-dependent loss of acid phosphatase activity and immunoprecipitable uteroferrin. Proteolytic cleavage also begins to occur. Fe on uteroferrin is transferred in a time-dependent manner to a protein with the properties of transferrin.

4. The polypeptide composition of allantoic fluid throughout gestation (Day 30 to Day 105) was analyzed by two-dimensional polyacrylamide gel electrophoresis. Major changes in the amounts of the proteins originating from the maternal endometrium (including uteroferrin) were noted during pregnancy. Levels of these proteins were maximal between Days 45 and 75 of pregnancy. Amounts were very low at Days 90 and 105. Many serum-like proteins were identified in allantoic fluid, including transferrin. Other than for the presence of uterine proteins in

allantoic fluid, the polypeptide composition of allantoic fluid, amniotic fluid, and fetal serum were very similar.

5. Uteroferrin, when labelled with ^{59}Fe and injected in vivo into the allantoic sac of Day 60 pregnant gilts, lost in an approximate first order manner its Fe with a half-life of about 15 hours. The ^{59}Fe was recovered largely in fetal transferrin in allantoic fluid and within the fetus, was found to be sequestered in fetal liver, spleen, and erythrocytes (in hemoglobin). Calculations indicate that at Day 60, loss of Fe from uteroferrin amounts to 1.12 - 1.89 mg/pregnant gilt.

6. Uteroferrin, when labelled with ^{125}I and injected in vivo into the allantoic sac of Day 60 pregnant gilts, does not appear to be found in equilibrium with fetal blood nor to be recovered in intact form from fetal cord blood.

7. Porcine transferrin, labelled with ^{59}Fe and injected in vivo into the allantoic sac, gave a distribution of Fe in fetal tissues similar to that of uteroferrin.

8. Porcine transferrin, labeled with ^{125}I and injected in vivo into the allantoic fluid, was recovered largely in intact form in both the allantoic fluid and fetal cord blood. The protein appears to pass readily from the allantoic sac into the fetal blood stream.

9. Model degradation studies using trypsin, chymotrypsin and allantoic fluid revealed two groups of fragments, A having a M_r of about 18,000 - 22,000 and B with a M_r of about 14,500, are generated indicating a possible protease sensitive region in uteroferrin. This cleavage occurred before Fe was lost from the protein. Apo-uteroferrin is much more rapidly degraded than Fe-containing uteroferrin and fragments A and B are not detected.

10. Iron release studies indicate that a low molecular weight chelator, such as ascorbate, ATP, pyrophosphate, and citrate, can mediate the release and transfer of Fe when a suitable Fe acceptor is present. Ascorbate appears to be the most likely candidate in allantoic fluid.
11. Results are consistent with the following model.

Uteroferrin is synthesized in the surface and glandular epithelium of the maternal uterus and is responsible for the transport of Fe to the developing fetus. Thus, transport is maximal between Days 45 and 90. Maternal transferrin is not transferred.

At least a portion of the uteroferrin accumulates in allantoic fluid. Here the iron is lost in a time-dependent manner to transferrin which can escape the allantoic sac in intact form and distribute the Fe to the fetal tissues. Possibly uteroferrin is also in equilibrium with the fetal circulation but is rapidly degraded once it enters by the liver. After Fe is removed from uteroferrin in allantoic fluid, the apo-protein is rapidly degraded.

APPENDIX

VALIDATION OF RADIOIMMUNOASSAY

1. Inter-assay Variability

	Uteroferrin Concentration		
	Expected	Observed	Number of Assays
a)	0.5 µg/ml	Mean 0.51	n = 6
		S.D. 0.08	
		C.V. 15.7	
b)	0.75 µg/ml	Mean 0.79	n = 4
		S.D. 0.09	
		C.V. 11.4	
c)	1.0 µg/ml	Mean 1.04	n = 3
		S.D. 0.08	
		C.V. 7.7	

2. Intra-assay Variability

	Uteroferrin Concentration		
	Expected	Observed	Number of Samples
a)	0.4 µg/ml	Mean 0.44	n = 5
		S.D. 0.03	
		C.V. 6.8	

Specificity

Standard curve in buffer and increasing amounts of unknown in allantoic fluid diluted in buffer were parallel with slopes of -1.3806 and -1.5719, respectively.

Abbreviations: S.D. = standard deviation
C.V. = coefficient of variation
r = correlation coefficient

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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